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The goal of this proposal is to attempt a novel strategy for the use of viruses in the delivery of anti-cancer genes to prostate cancer cells. Current methods involving the use of viruses to deliver therapeutic genes for the treatment of any disease are hindered by the inability to target viral agents specifically to diseased tissue or organs. Recently, a protein marker has been characterized on the surface of prostate cancer cells. This marker, called the prostate-specific membrane antigen (PSMA), is readily detectable on prostate cancer cells from a large percentage of patients with prostate cancer. The abundance of this marker on cancerous prostate cells allows such cells to be detected and discriminated from both normal prostate cells and the cells of other bodily tissues. We have sought to analyze the possibility of utilizing the PSMA on the surface of prostate cancer cells as a way to target viral gene therapy agents specifically to tumors of the prostate.

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Table of Contents

Annual Summary

The end of the one-year extension of this fellowship has come to completion. Since the end of the first year, we have made tremendous progress toward accomplishing our goal of creating a PSMA-specific viral delivery system. In the last year (the extended year) I have extended the technologies developed throughout this project into another novel method for targetable gene delivery.

In order to accomplish the initial goals, we were forced to make significant modifications to the original proposed plan. However, we have found that these modifications have resulted in the creation of a broadly powerful technology to aid in the targeting of any type of virus particle to any area of interest. We have applied this new technology to the targeting of PSMA-overexpressing cells, and have succeeded in attaining our originally stated aims. In the extended year of this project, we have gone further, and have used the core technology developed in the first two years to create a method of gene delivery that is both safer and more efficient than traditional methods.

The initial Statement of Work described a plan whereby antibodies against prostate cancer cells would be placed on the surface of retroviral particles (Spleen necrosis virus and ecotropic murine leukemia virus). Placement of these antibodies was postulated to result in cell-type specific infection of prostate cancer cells. The attachment chemistry that was proposed to be used involved the attachment of biotinylated antibodies to streptavidin, which is tetrameric. The streptavidin would then function as a bridge, connecting the antibody to a biotinylated virus particle. All of this proposed work

has been carried out, however a major modification made has been the type of biotin molecule used on the surface of viral particles. Initial experiments, described in the first Annual Summary, showed that biotinylation of viral particles resulted in a decrease in viral infectivity. Noting this, we sought to discover whether by using a photocleavable variety of biotin (commercially available through Pierce Chemicals), we would be able to restore infectivity to biotinylated particles by simply exposing the particles to 365 nm light. In principle, we sought to determine whether we could create viruses whose infectivity could be shut-off by biotinylation, but restored only when and where the virus was exposed to light (365 nm)-hence creating photoactivatable viral vectors. As shown in the Appendix, this strategy worked extremely well with amphotropic retroviral particles, and resulted in the publication of this technique in the journal Gene Therapy.

Subsequent to the creation of photoactivatable viral particles. We applied this novel technique to Adenoviral particles, which we felt possessed higher biochemical stability and would perhaps be more amenable to surface chemical modifications then retroviruses. This strategy of photoactivation of viral infectivity worked very well also with adenoviral particles, indicating that this novel technique possessed versatility. Utilizing these photoactivatable adenoviral vectors, we were able to demonstrate photo-specific infection of a subcutaneous tumor in a mouse model, simply by irradiating through the skin with intense 365 nm light (see Appendix). This work was published in *Chemistry and Biology* and a copy of that paper is enclosed with this report. Having created this novel technology, we then decided to go back to the original Statement of Work and determine whether this strategy could be applied and refined to allow for the specific infection of PSMA-overexpressing cells. The success of this experiment was

described in my last (final) official annual report. In the extended year, we have taken the core technique, that is, the biotinylation of adenoviral particles and advanced it into a new method of targeted gene delivery described below.

The biotinylation of adenoviral particles renders the particles the powerful and broad capacity of being able to attach to any streptavidin-containing molecule or medium. We sought to determine whether the attachment of biotin-conjugated adenovirus particles could be attached to streptavidin-coated microbeads, and whether such virus-bead conjugates could be used as infectious gene delivery vehicles. The advantages of such conjugates would include the generation of diffusion-limited viral complexes, due to the relatively massive weight of the microbeads preventing the normal movement of viral particles in solution. Because this idea did not involve any need for photoactivation, we chose to use a non-photocleavable biotinylation reagent, sulfo-NHS-LC-biotin (SNLB) (Pierce) in the process of virus modification. We treated adenoviral particles with varying concentrations of SNLB and found that concentrations of 0.02 mg/ml were capable of biotinylating virus particles in the absence of any effect of viral infectivity. After purifying the biotinylated adenoviruses, we combined them with 0.5 micrometer-diameter streptavidin-coated silica microbeads. These beads have a density much greater than water (1.95 mg/ml) and have a high capacity for binding to biotinylated macromolecules. Centrifugation of these microbead-virus mixtures resulted in the removal of all biotinylated virus particles from solution, indicating that the beads had great amounts of bound adenovirus associated with them. We purified these beads, and tested their ability to transduce target cells grown in culture. We tested an array of cell lines, ranging from highly permissive to adenoviral infection, to non-permissive to

adenoviral infection. We found that bead-virus conjugates possessed great infectivity. In fact, we have found that such conjugates are far more infectious on a viral particle/infectious unit basis than free virus alone. Interestingly, cells normally non-permissive to adenoviral infection (in our test, that cell line was Colo205, a colon cancer cell line), were fully infectable when the adenovirus was delivered in bead-conjugated form. On one moderately permissive cell line, we found that virus delivered in bead-conjugated form was as much as 43-fold more infectious than standard, free, unmodified virus. We hypothesize that the density of the microbead conjugates is forcing the virus-bead conjugates to sink onto the surfaces of cells, forcing them to be in close contact, thus facilitating the infectious process by limiting viral diffusion away from cells. Additionally, we found that the infection of cells by adenovirus-microbead conjugates was far more targetable due to the tremendously limited diffusion of the virus particles. We were able to apply bead-virus conjugates to plates of target cells in the shapes of well-defined letters, by simply "writing" with the pipette as the virus-bead slurry was applied. Additionally, when the microbeads used as virus attachment centers were magnetic in nature, we were able to direct the infection of cells to well-defined areas through the use of strategically placed magnets. All of these data have been published in the journal *Virology*, and a reprint is provided along with this report.

In principle, the major goal of our original proposal has been met. We have successfully generated a PSMA-specific delivery system for viral particles. A vast majority of our originally proposed strategy remained intact through the conclusion of this work (the use of biotin and streptavidin and biotinylated antibodies). Notably however, we have not utilized the original proposed strategy of modifying the tropism of spleen necrosis or ecotropic murine

leukemia viruses. We feel however, that the development of photoactivatable viral vectors has resulted in a far greater contribution to the field of gene therapy in general. Additionally, the development of virus-microbead conjugates demonstrates moreover, the power and potential of the core technology derived from this project, i.e. the biotinylation of viral (retroviral and adenoviral) particles.

The work accomplished as a result of this fellowship has resulted in 3 manuscripts (one in *Gene Therapy*, and *Chemistry & Biology*, and *Virology* each), and two news articles on its behalf: one published in Nature Medicine, and one in the journal Biophotonics (see appendix). Enclosed, please find copies of each publication, in addition to copies of each news article derived from the work funded by this fellowship.

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4. News article, Biophotonics, May 2001
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VIRAL TRANSFER TECHNOLOGY RESEARCH ARTICLE

Photoactivatable retroviral vectors: a strategy for targeted gene delivery

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We have explored a novel strategy for the targeting of retroviral vectors to particular sites or cell types. This strategy involves a method whereby the infectivity of a retroviral vector is neutralized by treatment of viral particles with a photocleavable, biotinylation reagent. These modified viral vectors possess little to no infectivity for target cells. Exposure of these modified viral vectors to long-wavelength UV light induces a reversal of the neutralizing, chemical modification resulting in restoration of infectivity to the viral vector. This infectivity 'trigger' possesses great potential, both as a research tool and as a novel tactic for the targeting of gene-transfer agents, since it would become possible to direct both the time and location of a viral infection in a versatile manner. Gene Therapy (2000) **7**, 1999–2006.

Keywords: *retrovirus; biotin; photocleavable; gene therapy*

Introduction

There has been considerable success in the construction of highly infectious amphotropic viral gene transfer vectors. Retroviral vectors possessing either the murine amphotropic envelope glycoprotein or the envelope glycoprotein G of vesicular stomatitis virus (VSV-G), in addition to those vectors based on either adeno-associated virus (AAV) or adenoviruses have been generated which possess transduction efficiencies of 10^8 infectious units/ml and greater. The high transduction efficiencies of these viral vectors make them ideal for use both as research tools and as *ex vivo* gene transfer reagents for gene therapy procedures. However, the *in vivo* use of such vectors is restricted because of the broad tropism that these viruses possess.[1,2]

A major challenge, for the use of viruses and viral vectors as biologically useful tools continues to be the ability to target virus-mediated gene transduction to particular areas or cell types of interest. Much of the effort to construct viral vectors with targetable infectivity has involved the genetic modification of viral envelope glycoproteins, most commonly through the fusion of such proteins with binding reagents for particular cell types, such as single-chain antibodies, peptides, and ligands that can bind to cell-surface molecules.[3–5] Largely, this strategy has proven both unsuccessful and difficult. The genetic manipulation of all retroviral envelope glycoproteins tested thus far has shown that these glycoproteins are highly sensitive to alteration. Fusions of retroviral envelope glycoproteins with a variety of protein species have resulted in the generation of unstable, incorrectly folded, or non-functional proteins. In some cases, targetability to particular cell types was accomplished, but the resulting viral vectors showed markedly reduced transduction efficiencies.

Here, we describe an alternative strategy for the targeting of the infectivity of viral vectors. This strategy uses retroviral vectors, the infectivity of which is inhibited by a chemical modification that can be reversed upon exposure to light of a specific wavelength.

Results

We have explored an alternative strategy for the targeting of viral vectors to particular cell types or sites. This strategy involves a method whereby the infectivity of a viral vector is neutralized by a reversible chemical modification (Figure 1). Such chemically modified viral vectors possess little to no infectivity for potential target cells until an external stimulus is applied. This external stimulus induces a reversal of the neutralizing, chemical modification resulting in a restoration of infectivity to the viral vector. Such an infectivity trigger would possess great potential, as both a research tool and a targetable gene transfer agent, as it would become possible to direct both the time and location of a viral infection in a versatile manner. To test if this strategy can be employed to control the infectivity of retroviral vectors, we used a Moloney murine leukemia virus (MLV)-derived vector. This vector, termed Tel-Ampho, bears the envelope glycoprotein of the 4070A amphotropic murine retrovirus and packages a transducable, bacterial *lacZ* gene allowing for the easy identification and quantification of infected cells.[6] We tested NHS-PC-LC-Biotin as a modification reagent for the control of infectivity of Tel-Ampho. NHS-PC-LC-Biotin is a biotin derivative containing an *N*-hydroxysuccinimide ester (NHS) which reacts with primary amino groups by nucleophilic attack. This allows for simple attachment of the biotin moiety to the N-ter-

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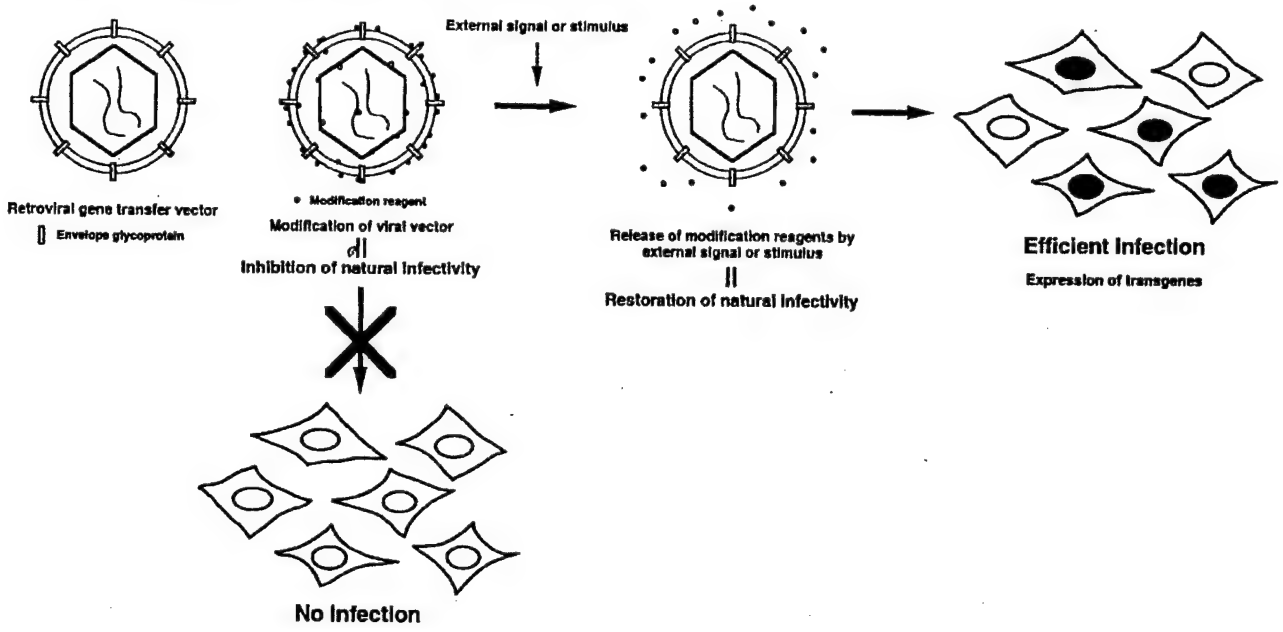


Figure 1 Concept of inactivation and subsequent reactivation of the infectivity of viral vectors using an inactivating reagent and an external (reactivating) stimulus.

mini and lysine residues of protein molecules. This biotin derivative also contains a photocleavable, 1-(2-nitrophenyl)ethyl moiety, which exhibits efficient cleavage upon exposure to light of wavelengths between 300 and 365 nm. Exposure of proteins, conjugated with NHS-PC-LC-Biotin to 300–365 nm light results in the release of the biotin moieties from the proteins, the conjugation sites (primary amino groups) of which are restored to their original, unmodified form.[7]

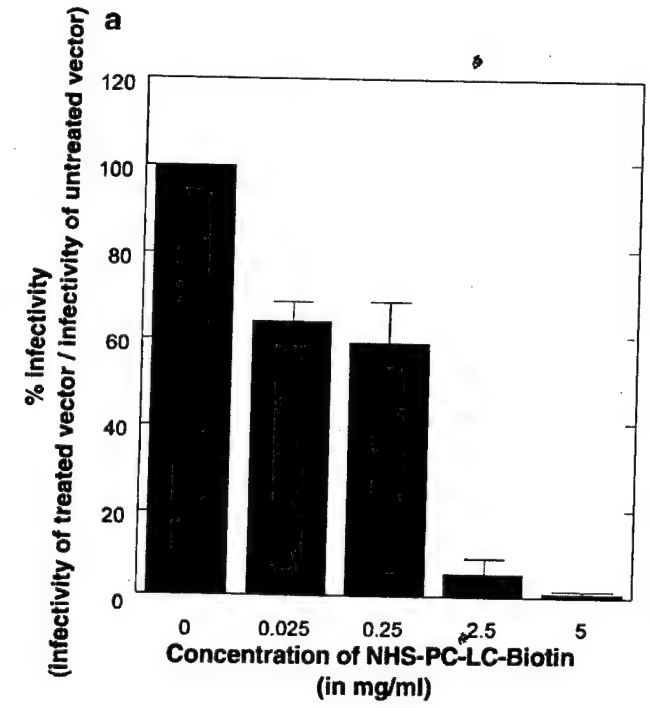
Treatment of Tel-Ampho with NHS-PC-LC-Biotin at concentrations of 2.5 and 5 mg/ml, greatly reduced the infectivity of this viral vector (Figure 2a). In some cases, at these concentrations, the infectivity of Tel-Ampho was completely eliminated. The presence of biotinylation reagent on viral vector components was implied by the marked effect of the reagent on the infectivity of Tel-Ampho. To confirm that the inhibition of infectivity caused by the treatment of NHS-PC-LC-Biotin was due to conjugation of this reagent to virions, and not to the presence of unreacted reagent, Tel-Ampho reacted with NHS-PC-LC-Biotin was combined with excess glycine that efficiently reacts with unreacted NHS-PC-LC-Biotin. Additionally, this post-conjugation treatment with excess glycine was also performed after the removal of unreacted NHS-PC-LC-Biotin from viral vectors by several rounds of ultrafiltration. However, neither of these treatments showed any effect on the infectivity inhibition of Tel-Ampho by treatment with NHS-PC-LC-Biotin. These results, along with the fact that the NHS moiety of NHS-PC-LC-Biotin undergoes rapid hydrolysis in aqueous media[8,9] suggest that the infectivity inhibition of Tel-Ampho by NHS-PC-LC-Biotin is caused by conjugation of the biotinylation reagent to viral vectors.

We next tested if the infectivity of Tel-Ampho, which has been inhibited by treatment of NHS-PC-LC-Biotin, could be restored by exposure to 365 nm light. This wavelength of light can cleave NHS-PC-LC-Biotin away from proteins to which it has been conjugated and would cause minimal damage to retroviral particles. Because photocleavage of this reagent results in the original target molecule being restored to its unmodified form, we reasoned that exposure of retroviral vectors treated with NHS-PC-LC-Biotin might regain their infectivity. We treated three different stocks of Tel-Ampho with 2.5 mg/ml NHS-PC-LC-Biotin, which resulted in nearly complete inhibition of their infectivity. These retroviral vectors were then exposed to 365 nm light and at regular intervals, samples of these treated stocks were collected and analyzed for their ability to infect target cells (Figure 2b). Each of the biotinylated Tel-Ampho stocks showed demonstrative gains in infectivity upon exposure to 365 nm light. Restoration of infectivity occurred within 4–6 min of irradiation. As shown for stock 3, irradiation of biotinylated viral vector resulted in nearly complete restoration of the infectious potential for the viral vector stock. Exposure of viral vectors to 365 nm light for longer than 8 min had a detrimental effect on viral infectivity. This might be caused by the damage of viral components by shorter-wavelength UV light that would also be emitted by the light source used. These data indicate that the inhibition of infectivity caused by the reaction of Tel-Ampho vectors with NHS-PC-LC-Biotin can be reversed upon exposure of these biotinylated, inactivated retroviral vectors to 365 nm light. These data indicate that these retroviral vectors, when treated with NHS-PC-LC-Biotin, possess light-activatable infectivity. A series of experiments showed that the infectivity of Tel-Ampho was restored to 30–90% of the original infectivity upon photo-irradiation.

We next sought to determine whether activation of retroviral vectors could be performed within the context of virus target cells. To investigate this, Tel-Ampho was treated with 2.5 mg/ml NHS-PC-LC-Biotin as described earlier. Equal amounts of biotinylated Tel-Ampho were

then added to cultures of D-17 cells,[10] followed by either irradiation with 365 nm light for 5 min or placement in the dark. As shown in Figure 3, target cells mixed with biotinylated and nonirradiated viral vectors showed little or no infection. In contrast, those mixtures of viral vectors and cells that had been exposed to 365 nm light showed considerable infection of target cells. No detectable difference in cell viability or growth rate was observed between the irradiated and nonirradiated cultures, indicating that neither the exposure of long-wavelength UV light nor unreacted biotinylation reagent, if present, had any effect on cell growth or viability. These data demonstrate that the infectivity of these viral vectors can be activated *in situ*. This offers the potential for the use of this strategy to target viral vector infection at a location of interest by using focused light.

To determine which viral components were biotinylated by treatment with NHS-PC-LC-Biotin, we analyzed biotinylated, inactivated Tel-Ampho using Western blotting analysis. Concentrated Tel-Ampho was treated with 2.5 mg/ml NHS-PC-LC-Biotin as above. The resulting Tel-Ampho samples were subject to gel filtration chromatography using Sephacryl S-1000 followed by passage through a 0.45-μm filter and subsequent centrifugation (to purify virions from other materials).[11] The resulting viral vectors were either irradiated with 365 nm light or kept in a dark place before the separation of viral components by SDS-PAGE.[12] Biotinylated viral components were identified by using a streptavidin-peroxidase conjugate as a probe after transfer to PVDF membrane (Figure 4a). Biotinylated proteins are found at approximately 150, 30, 25 and 15 kDa on the blot (lane B). The molecular masses of these protein species suggest that they are the *gag-pol* and *gag* protein products of MLV.[13] This is supported by the fact that these biotinylated viral components comigrate with proteins that are immunoreactive with polyclonal antibody against disrupted murine leukemia virus particles (lane A). In contrast, the Tel-Ampho that was treated with NHS-PC-LC-Biotin and subsequently subjected to irradiation with 365 nm light shows markedly less binding by the streptavidin-peroxidase conjugate (lane C). In particular, the viral components of approximately 150, 25 and 15 kDa, seen in nonirradiated Tel-Ampho (lane B), are absent in the lane containing an equal amount of irradiated virus (lane C). However, a biotinylated viral component of approximately 30 kDa remains readily detectable, although the signal intensity is reduced. This indicates that removal of NHS-PC-LC-Biotin from this viral component did not occur to completion under the exposure conditions used. From its molecular mass, this protein species is the core (*gag*) antigen of MLV. The location of this protein in virions at the viral core may render it less susceptible to photon irradiation, compared with matrix (p15) or *gag-pol* (approximately 200 kDa) components, which are membrane-associated and thus should be readily access-



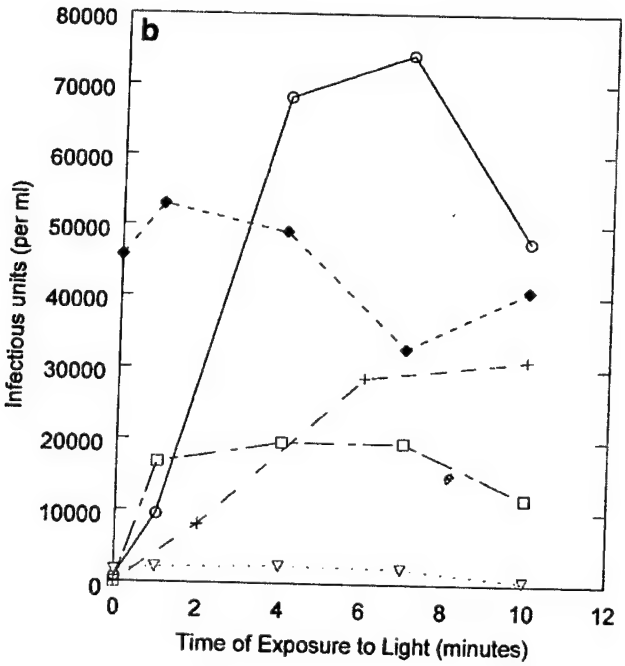


Figure 2 (a) Effect of treatment with NHS-PC-LC-Biotin on the infectivity of Tel-Ampho. Serial dilutions of 25 mg/ml NHS-PC-LC-Biotin were made in dimethylformamide (DMF) and were allowed to react with aliquots (200 μl) of Tel-Ampho for 150 min before halting the reaction and was followed by viral infectivity assay (as described in Materials and methods). DMF was found to have no effect on virion infectivity at dosages up to 30% (data not shown). Data shown are the average of two experiments and is representative of at least three independent experiments. (b) Time course of the restoration of viral infectivity to biotinylated, inactivated Tel-Ampho upon exposure to long-wavelength (365 nm) UV light. Tel-Ampho (200 μl) was treated with 2.5 mg/ml NHS-PC-LC-Biotin for 150 min on ice as in panel a. Treated viral stocks were placed in borosilicate glass vials and subjected to irradiation with long-wavelength (365 nm) UV light (at a distance of 0.5 cm, utilizing a UVL-21 lamp, intensity of 720 μW/cm² at 15 cm distance) (UV Products). At the intervals shown, samples (10 μl) of viral vectors were removed from vials and placed over monolayers of D-17 cells for infectivity analysis. ○, Stock 1, biotinylated, irradiated; □, stock 2, biotinylated, irradiated; +, stock 3, biotinylated, irradiated; ◆, stock 3, not biotinylated, irradiated; ▽, stock 3, biotinylated, not irradiated.

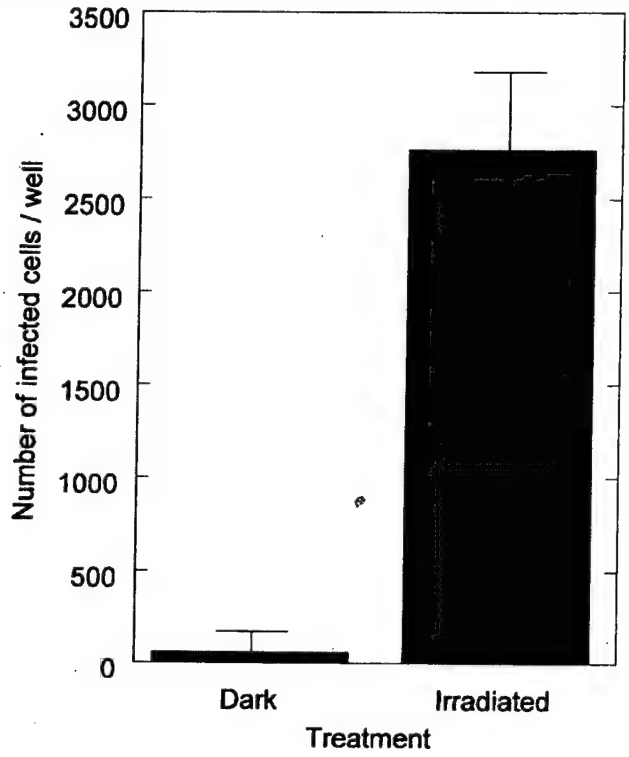


Figure 3 Light-activated infection of D-17 cells. Tel-Ampho (50 μl), concentrated as described in Materials and methods and subsequently treated with 2.5 mg/ml NHS-PC-LC-Biotin for 150 min, was placed within each of six 35-mm culture dishes containing monolayers of D-17 cells (2×10^5/dish) covered with 500 μl of DMEM/10% FBS supplemented with 5 μg/ml polybrene. Three of the dishes were placed on ice in a dark box, while the remaining three dishes were placed on ice and exposed to 365 nm light. Exposure was from the top of open culture dishes and was performed for 6 min at a distance of 2 cm between the source (UVL-21 lamp) and the monolayer of cells. Exposure was for 6 min. At 48 h after irradiation, cells were then subjected to infectivity assays, as described in Materials and methods.

ible to light. These data suggest that biotinylation of the core *gag* antigen is not the cause of infectivity inhibition by treatment with NHS-PC-LC-Biotin. Also notable is the apparent lack of detectable signal for the gene products of *env* (80 kDa) and *pol* (80 kDa). This may be caused by the lack of sensitivity in this analysis, since these viral components exist in much smaller amounts than the *gag* gene products in mature virion particles.[13] Thus, from this analysis, we are unable to determine whether or not the gene products of *env* and *pol* are biotinylated by treatment with NHS-PC-LC-Biotin.

The effect of biotinylation on the reverse transcriptase (RT) (a *pol* gene product) activity of Tel-Ampho particles was determined by RT assays. Tel-Ampho was treated with NHS-PC-LC-Biotin and divided into two samples, one irradiated with 365 nm light and the other kept in the dark. RT assays on these samples after disruption of virions (Figure 4b) show that biotinylation of Tel-Ampho with NHS-PC-LC-Biotin resulted in an approximate 50% decrease in RT activity. However, exposure of the biotinylated Tel-Ampho to 365 nm light had no significant effect on the RT activity. These data indicate that the RT activity was reduced by treatment of Tel-Ampho, suggesting biotinylation of virion-associated RT. However, because the reduction of RT activity caused by NHS-PC-LC-Biotin was not restored by irradiation, it is unlikely that modulation of RT activity can account for the infectivity inhibition by biotinylation and its restoration upon light irradiation.

Although the biotinylation of the *env* gene product was not apparent by Western blotting analysis (Figure 4a), it remains possible that this viral component was modified by biotinylation. To assess whether the envelope glycoprotein of Tel-Ampho was affected by biotinylation, a viral interference assay was performed. This assay is designed to assess the ability of a set of viral particles to inhibit the binding and entry of another set of viral particles by competing for interaction with the cell-surface receptors used for viral infection. First, the ability of this assay to assess the function of the *env* gene product was determined. Tel-Ampho vector was concentrated and subsequently inactivated by a psoralen derivative, 4'-aminomethyl-4,5',8-trimethylpsoralen (AMT), and exposure to long-wavelength UV light. Inactivation of Tel-Ampho by the psoralen derivative is caused by cross-linking of the viral RNA genomes and has no significant effect on other properties of the viral vectors, including the binding to target cells.[14–16] These psoralen-inactivated Tel-Ampho particles were mixed with unmodified Tel-Ampho vector to see if they can inhibit the infectivity of unaltered Tel-Ampho for target cells. Psoralen-inactivated Tel-Ampho, when in a ratio of approximately 20:1 with unaltered Tel-Ampho, strongly inhibited infection of target cells by the unaltered Tel-Ampho (Figure 4c). Identical viral cores which display the envelope glycoprotein of spleen necrosis virus (SNV), Tel-SNV, were tested in the same manner. The psoralen-inactivated Tel-SNV was markedly ineffectual relative to psoralen-inactivated Tel-Ampho in inhibiting the infection of target cells by unaltered Tel-Ampho. These results indicate that the inhibition of Tel-Ampho infection by psoralen-inactivated Tel-Ampho was caused primarily by inhibition of the ability of the *env* gene product to mediate the binding and/or entry to target cells.

Tel-Ampho was inactivated by psoralen treatment as above, and then biotinylated with NHS-PC-LC-Biotin. The resulting psoralen-inactivated, biotinylated Tel-Ampho was tested for the ability to inhibit the infection of target cells by unaltered Tel-Ampho. The psoralen-inactivated, biotinylated Tel-Ampho was demonstrably less effective than psoralen-inactivated, nonbiotinylated Tel-Ampho in blocking the infection of target cells by unaltered Tel-Ampho. When 10^4 target cells were used, psoralen-inactivated, biotinylated Tel-Ampho was approximately 45-fold less effective at inhibiting the infectivity of unaltered Tel-Ampho than their inactivated, nonbiotinylated counterpart. These data imply that biotinylation of Tel-Ampho with NHS-PC-LC-Biotin results in a loss of the function of the envelope glycoprotein. Therefore, it is possible that the infectivity inhibition caused by NHS-PC-LC-Biotin treatment is due to the inability of biotinylated viral vectors to bind to and/or to enter target cells. The possibility of this as a mechanism of infectivity inhibition is supported by the observation that a similar biotinylation reagent, which is charged and highly water-soluble (sulfo-NHS-LC-Biotin; Pierce Chemical), inactivated the infectivity of Tel-Ampho at identical concentrations as NHS-PC-LC-Biotin (data not shown). This water-soluble biotinylation reagent is incapable of pass-

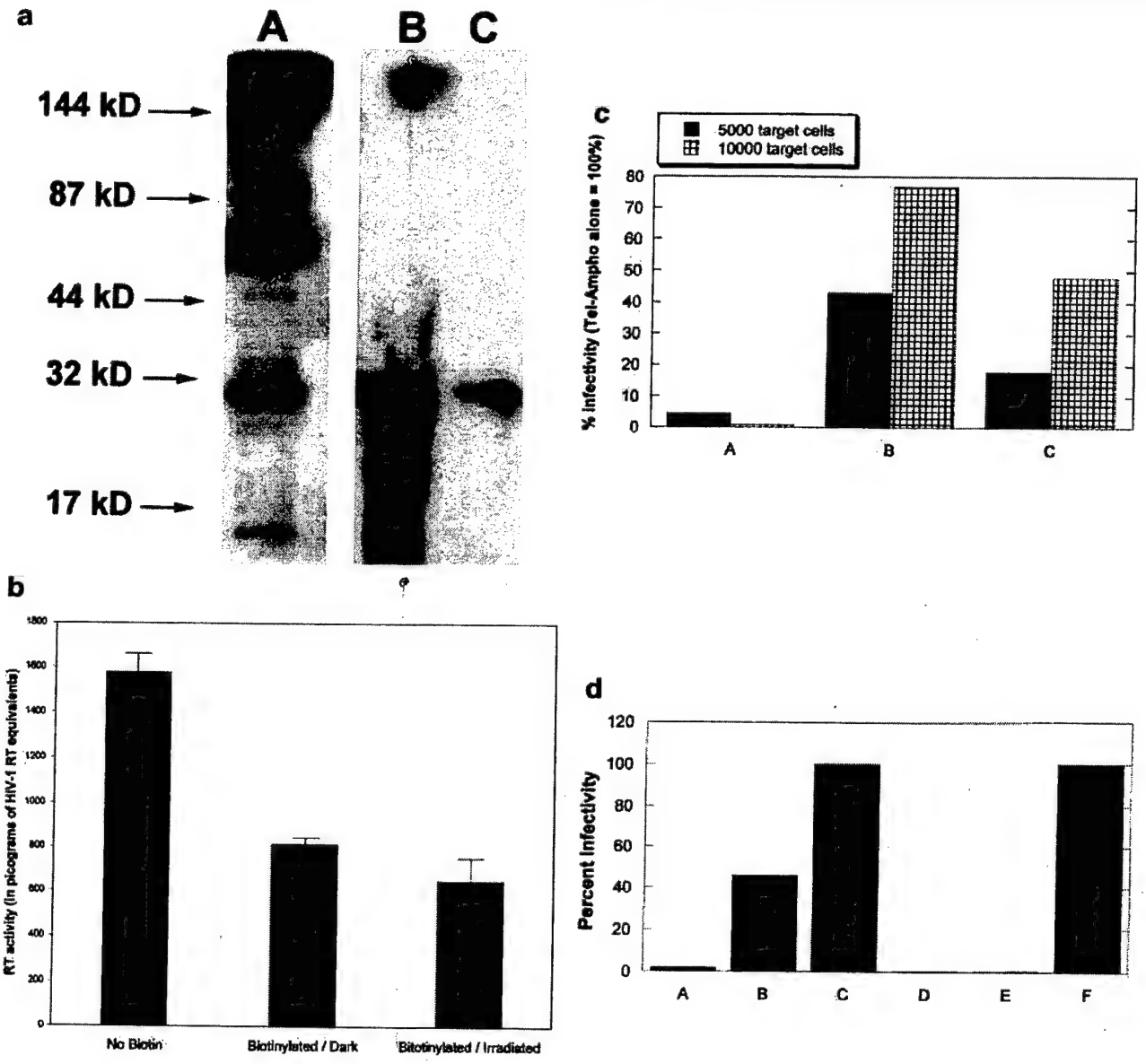


Figure 4 (a) Western blotting analysis of biotinylated, purified Tel-Ampho. Lane A, Western blot of lysed Tel-Ampho, detected with polyclonal antiserum raised against disrupted MLV. The data shown are representative of two experiments. (b) Effect of biotinylation on reverse transcriptase activity of Tel-Ampho. (c) Analysis of the effect of biotinylation on the function of the env gene product using a viral interference assay. A, Tel-Ampho with psoralen-inactivated Tel-Ampho; B, Tel-Ampho with psoralen-inactivated Tel-SNV; C, Tel-Ampho with psoralen-inactivated, biotinylated Tel-Ampho. (d) Comparison of Tel-Ampho with Tel-SNV for use as light-activatable viral vectors. A, Tel-Ampho, biotinylated with 2.5 mg/ml NHS-PC-LC-biotin, stored in the dark; B, Tel-Ampho, biotinylated as A, and subsequently exposed to 365 nm light for 3 min; C, Tel-Ampho, treated with an equivalent amount of DMF as in A and B (with no biotinylation reagent), stored in the dark; D, Tel-SNV, biotinylated as A, stored in the dark; E, Tel-SNV, biotinylated as D, and subsequently exposed to 365 nm light for 3 min; F, Tel-SNV, treated with an equivalent amount of DMF as D and E (no biotinylation reagent), stored in the dark. Data shown are representative of two independent experiments.

ing through lipid bilayers.[17] Hence, it should biotinylate only the outer surface of (enveloped) retroviral particles.

Also supporting the role of the envelope glycoprotein in biotinylation-mediated inactivation of infectivity is the observation that Tel-SNV (which is otherwise identical to Tel-Ampho except that it bears the envelope glycoprotein of SNV instead of that of amphotropic MLV) showed a behavior very different from Tel-Ampho when treated with NHS-PC-LC-Biotin. While the infectivity of Tel-SNV was effectively inactivated by treatment with NHS-PC-LC-Biotin, exposure of the resulting biotinylated Tel-SNV with 365 nm light did not restore viral infectivity (Figure 4d). Because the only difference between the Tel-Ampho and Tel-SNV particles is the type of envelope glycoprotein displayed, it is implied that the phenomenon of light-facilitated infectivity activation involves the inactivation and subsequent reactivation of the function of the viral envelope glycoprotein.

Discussion

Here we have demonstrated the potential for the creation of viral vectors with externally activatable infectivity. Using a photocleavable reagent and simple procedures, the infectivity of an amphotropic retroviral vector species can be nearly or completely eliminated. Upon exposure of these viral vectors to light, infectivity was restored to these viral vectors. This strategy of activatable infectivity worked in either the presence or absence of viral target cells, demonstrating its ability to provide effective control over the transduction of genes by amphotropic viral vectors. Possible applications of viral vectors with activatable infectivity are numerous. For example, the infectivity of amphotropic vectors, normally not targetable due to the lack of tissue or cell-type specificity, could conceivably be activated within specific areas by a directed, focused application of light. Because treatment with NHS-PC-LC-Biotin results in the attachment biotin moieties to retroviral vectors, it may be possible to conjugate biotin-binding proteins, such as avidin and streptavidin to these biotinylated and inactivated vectors. Streptavidin could function as a bridge to conjugate a variety of targeting reagents, such as single-chain antibodies against cell-surface molecules of target cells, to the surface of biotinylated and inactivated viral vectors. Retroviral vectors with activatable infectivity may also be useful as a research tool, where either temporal or spatial control of an infection is desired. Additionally, the ability to switch infectivity off or on might be desirable for safe handling of highly contagious or dangerous pathogens. The transportation of such pathogens might also be made safer if their infectivity could be inactivated before transport, to be restored upon reception.

We are finding that a number of human cell lines behave similarly to the D-17 line (data not shown). However, the current treatment concentration (2.5 mg/ml) of NHS-PC-LC-Biotin resulted in varied levels of inactivation from cell line to cell line. This suggests that optimization of treatment conditions may be required for each cell type in question.

Materials and methods

Cell lines and virus production

The dog osteosarcoma cell line, D-17 (10) (American Type Culture Collection, Manassas, VA, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM; Cellgro, Herndon, VA, USA) supplemented with 6% fetal bovine serum and penicillin-streptomycin. A human rhabdomyosarcoma cell line, TelCeB6,[3] was maintained in DMEM supplemented with 10% FBS and penicillin-streptomycin.

For production of retroviral stocks bearing the amphotropic envelope from 4070A murine leukemia virus (Tel-Ampho), TelCeB6 cells (10^7) were cultured overnight in 150-mm culture dishes. These cells produce murine leukemia virus particles that possess *gag* and *pol* gene products, but lack envelope glycoprotein. Cells were transfected by the calcium-phosphate method with 20 μg of the plasmid, pA,[3] which expresses the amphotropic envelope glycoprotein of 4070A murine leukemia virus. After transfection (72 h), culture media containing Tel-Ampho retroviral vectors were collected, and centrifuged at 4000 *g* for 10 min. Supernatants were then divided into aliquots and stored at –70°C. Production of retroviral vectors bearing the spleen necrosis virus (SNV) envelope glycoprotein was accomplished by the method described above, using a plasmid, pRD134, that efficiently expresses the envelope glycoprotein of SNV.[5]

Infectivity assays

To determine the infectivities of retroviral vectors, virus particles were placed over monolayers of D-17 cells (10 000 per well, in a 96-well plate, or 40 000 per well in a 24-well plate) in a total volume of 100 μl or 200 μl, respectively, of DMEM/10% FBS containing 5 μg/ml polybrene. After exposure of retroviral vectors to D-17 cells (48 h), cells were fixed in 0.5% paraformaldehyde and stained for the expression of *lacZ* gene using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as the substrate. Infected (*lacZ*-expressing) cells, which possessed easily identifiable blue nuclei, were counted under a light microscope.

Biotinylation of viral vectors and light exposure of biotinylated viral vectors

Serial dilutions of 25 mg/ml NHS-PC-LC-Biotin (Pierce Chemical, Rockford, IL, USA) were made in dimethylformamide (DMF). Dilutions of NHS-PC-LC-Biotin were added to aliquots (200 μl) of Tel-Ampho stock solutions, and the final concentration of DMF in each treated vector stock was adjusted to 10% (v/v). NHS-PC-LC-Biotin and Tel-Ampho mixtures were placed on ice and allowed to react in the dark for 150 min. Reactions were terminated by the addition of 200 μl of 100 mM glycine/PBS (pH 7.4) and 200 μl of DMEM containing 10% FBS and 30 min of storage at 4°C. Samples of NHS-PC-LC-Biotin-treated viral vectors (10 μl) were then placed over monolayers of D-17 (10 000 per well in a 96-well plate) and subject to infectivity assays.

For analysis of the time-course of biotinylated virion infectivity *versus* irradiation with long-wavelength UV light, a Tel-Ampho stock solution (2 ml) was subject to ultrafiltration to a final volume of 200 μl using filtration devices (YM-100, Millipore, Bedford, MA, USA) with a molecular mass cut-off of 100 kDa. This was followed by dilution of the concentrated viral vector with PBS (pH 7.4) to a volume of 2 ml. This process was repeated, and the resulting viral vector stock was concentrated to a volume of 200 μl by ultrafiltration. Concentrated viral vectors were then treated with 2.5 mg/ml NHS-PC-LC-Biotin for 150 min on ice. Treated viral stocks were placed in borosilicate glass vials and subject to irradiation with long wavelength (365 nm) UV light at a distance of 0.5 cm, utilizing a UVL-21 lamp (720 μW/cm² at 15 cm distance) (UV Products, Upland, CA, USA). At the intervals shown, samples (10 μl) of virus were removed from vials and subjected to infectivity assays, as described above.

Western blotting analysis

Tel-Ampho (3 ml) was filtered by passage through a 0.45-μm filter. Filtered viral vector was centrifuged at 8000 *g* for 10 min at 4°C. The supernatants were concentrated to a volume of 200 μl by YM-100 ultrafiltration (100 kDa molecular mass cut-off). Viral vectors were treated with 2.5 mg/ml NHS-PC-LC-Biotin/10% DMF for 150 min on ice in the dark. An equal volume of 100 mM glycine/PBS (pH 7.4) was added to the biotinylated viral vectors, and the mixture was placed on ice, in the dark for 30 min.

The treated viral vectors were subjected to ultrafiltration to reduce the volume to 200 μl. This concentrated, biotinylated viral vector was placed in a 2.5-ml (7.5-cm length) Sephacryl S-1000 gel filtration chromatography column. Viral vectors were eluted with PBS (pH 7.4), and fractions from 400 μl to 1500 μl were collected (determined to be the virus-containing fractions). Purified viral vector (500 μl) was stored in the dark at room temperature or exposed to irradiation by 365-nm light for 6 min at a distance of 0.5 cm (as described above). These viral vectors were centrifuged at 24 000 *g* at 4°C for 2 h to pellet the viral vector particles. Pelleted viral vectors were suspended in 15 μl of an SDS-containing sample buffer and subjected to SDS-PAGE[12] using 10–20% polyacrylamide gradient gels. Proteins were blotted on PVDF membrane that was then blocked with SuperBlock (Pierce). Blots were incubated for 1 h in a 1/10 000 dilution of a streptavidin-peroxidase conjugate (Pierce) in SuperBlock containing 0.02% Tween 20. Binding of the streptavidin-peroxidase conjugate was detected by using an enhanced chemiluminescence (ECL) kit (Amersham, Piscataway, NJ, USA). To detect all viral components on blots, Tel-Ampho (1 ml) were subjected to ultrafiltration using a YM-100 filtration unit to a final volume of 100 μl. The concentrated viral vectors were subjected to albumin removal using an albumin serum removal kit (Genomic Solutions, Ann Arbor, MI, USA) following the protocol as described by the manufacturer. The resulting viral vector solution was centrifuged at 24 000 *g* for 2 h at 4°C. The viral pellet was dissolved in 15 μl of an SDS-containing sample buffer, and 5 μl of the resulting lysed virus was loaded into the same gel and blotted on to the same membrane as above. The blot was then blocked as described above and subsequently incubated with a 1/10 000 dilution of goat polyclonal antisera against disrupted Rauscher leukemia virus (ID 71S000126, NCI/BCB Repository), which crossreacts with the protein components of Moloney murine leukemia virus. Blots were then treated with a 1/100 000 dilution of sheep anti-goat polyclonal antibodies conjugated to peroxidase and subjected to ECL for visualization.

Reverse transcriptase assays

Tel-Ampho vector (6 ml) was pelleted by centrifugation at 24 000 *g* for 2 h at 4°C. The viral pellet was resuspended in 130 μl of PBS (pH 7.4). Concentrated viral vector (80 μl) was then treated with 2.5 mg/ml NHS-PC-LC-Biotin/10% DMF for 150 min on ice, in the dark. In parallel, 40 μl of the concentrated viral vector was with 10% DMF alone for 150 min on ice, in the dark. NHS-PC-LC-Biotin-treated viral vector (40 μl) was exposed to 365-nm light for 6 min at a distance of 0.5 cm as described above. The other 40 μl of biotinylated vector was allowed to stand in the dark at room temperature for 6 min. All viral vectors were then divided into 20 μl aliquots, each mixed with 20 μl of a lysis buffer (50 mM Tris-Cl, 80 mM potassium chloride, 2.5 mM DTT, 0.75 mM EDTA, and 0.5% Triton X-100, pH 7.8). Lysed viral vectors were then combined with 50 μl of a reverse transcriptase reaction buffer containing deoxyribonucleotides and template RNA (provided by the Non-Radioactive Reverse-Transcriptase Assay Kit, Boehringer Mannheim, Indianapolis, IN, USA). Manganese chloride was added to a final concentration of 12 mM. α-^{32}P-dTTP, 7 μl, was also added to allow for detection of RT reaction products. Lysed viral vectors mixed with the reaction buffer were incubated at 42°C and incubated overnight. Each reaction mixture (10 μl) was spotted on DE81 filter paper (Whatman, Clifton, NJ, USA), dried, and washed three times with 2 × SSC. After washing, the spotted filter papers were dried after soaking them in 95% ethanol and radioactivity was assessed by liquid scintillation counting.

Viral interference assays

Inactivated Tel-Ampho particles were prepared by treating a stock of Tel-Ampho with a psoralen derivative, AMT, followed by irradiation with long-wavelength UV light. These psoralen-inactivated viral vectors were tested for their ability to interfere with the infection of viral target cells (D-17) by unaltered Tel-Ampho. In parallel, Tel-SNV (TelCeB6-derived viral cores bearing the envelope glycoprotein of spleen necrosis virus) and NHS-PC-LC-Biotin-treated Tel-Ampho (using 2.5 mg/ml NHS-PC-LC-Biotin) were also tested for their ability to interfere with the ability of unaltered Tel-Ampho vectors to infect target D-17 cells. Stocks of inactivated Tel-Ampho and Tel-SNV particles were constructed as follows. The viral vector stock (1.6 ml) was combined with 4′-aminomethyl-4,5′,8-trimethylpsoralen (AMT) at a final concentration of 50 μg/ml. After 30 min of incubation at room temperature in the dark, viral vector-AMT mixtures were exposed to 365 nm for 15 min at a distance of 2 cm from the light source. Following irradiation, AMT-inactivated viral vector was centrifuged at 8000 *g* for 10 min at 4°C to remove gross cellular debris. Virus-containing supernatants were then concentrated to a volume of 420 μl by ultrafiltration using YM-100 (100 kDa molecular mass cut-off) filtration devices. Concentrated viral vector was separated into two 200-μl aliquots and treated with either 2.5 mg/ml NHS-PC-LC-Biotin/10% DMF or 10% DMF alone, on ice in the dark for 150 min, followed by addition of 200 μl of 100 mM glycine/PBS (pH 7.4). Inactivated, concentrated and NHS-PC-LC-Biotin-treated (or non-biotinylated) viral vector (20 μl) was then combined with 2.5 μl of unaltered Tel-Ampho from the same viral vectors stock as the one used to prepare inactivated viral particles. These mixtures were placed over monolayers of either 1×10^4 or 5×10^3 D-17 cells in a total volume of 100 μl of DMEM/6% FBS containing 5 μg/ml polybrene. After 4 h of incubation at 37°C, the culture medium over the D-17 cells was replaced with fresh DMEM/6% FBS. After exposure of cells to viral vectors (48 h), cells were fixed in 0.5% paraformaldehyde and stained with X-gal to reveal infected (*lacZ*-expressing) cells as described above.

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Photochemical Control of the Infectivity of Adenoviral Vectors Using a Novel Photocleavable Biotinylation Reagent

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Summary

We have explored a novel strategy for controlling the infectivity of adenoviral vectors. This strategy involves a method whereby the infectivity of adenoviral vectors is neutralized by treatment of viral particles with a water-soluble, photocleavable biotinylation reagent. These modified viral vectors possess little to no infectivity for target cells. Exposure of these modified viral vectors to 365 nm light induces a reversal of the neutralizing, chemical modification, resulting in restoration of infectivity to the viral vectors. The light-directed transduction of target cells by photoactivatable adenoviral vectors was demonstrated successfully both in vitro and in vivo. This photochemical infectivity trigger possesses great potential, both as a research tool and as a novel tactic for the delivery of gene-transfer agents, since the infectivity of adenoviral vectors can be controlled externally in a versatile manner.

Introduction

Adenoviral vectors have become one of the most useful gene transfer agents [1–5]. They are structurally stable and can be produced to high infectious titers (up to $\sim 10^{12}$ infectious units/ml). Their genome can be easily manipulated to deliver large transgenes. Adenoviral vectors can infect a wide range of cells, including nondividing cells. These characteristics make adenoviral vectors a particularly attractive tool for a wide variety of gene transfer applications. However, the broad target cell range of adenoviral vectors has two consequences that limit their utility for in vivo gene transfer applications, such as gene therapy. First, their broad tropism can result in the delivery of the transgene in a nonspecific manner. Second, large doses of undirected adenoviral vectors would be required to ensure an adequate amount of gene transfer to the target site. Thus, the ability to control the delivery of adenoviral vectors in a controlled or directed manner is of paramount importance in the development of adenovirus-based in vivo gene transfer protocols.

We have explored a novel strategy for controlling the infectivity of adenoviral vectors. This strategy employs a chemical agent that modifies adenoviral vectors in a reversible fashion such that their infectivity is eliminated but can be restored upon application of an external stimulus. This strategy allows adenovirus-mediated gene transduction to be externally controllable.

Results and Discussion

We have created a novel strategy for controlling the infectivity of adenoviral vectors. This strategy involves a method whereby the infectivity of an adenoviral vector is first neutralized by a reversible chemical modification. Reversal of this chemical modification allows for the restoration of infectivity to the adenoviral vector. This strategy had recently been successfully implemented on retroviral vectors derived from amphotropic Moloney murine leukemia virus by using photocleavable biotin (PCB) [6] as the modification reagent [7]. PCB (Figure 1A) contains a biotin moiety linked though a spacer arm to a 1-(2-nitrophenyl)ethyl group, which is derivatized with an NHS (*N*-hydroxysuccinimide) ester. The NHS ester reacts selectively with primary aliphatic amino groups, such as N termini and lysine residues on proteins, which are abundant in viral vectors, to form a carbamate bond. When PCB-biomolecule conjugates are exposed to 300–365 nm light, the PCB moiety undergoes an intramolecular photochemical reaction, which involves the cleavage of the carbamate bond. This results in the regeneration of the primary amino group on the biomolecule and releases the PCB moiety, in which the 1-(2-nitrophenyl)ethyl group is converted to a 2-nitrosoacetophenone derivative [6]. PCB is highly hydrophobic and exhibits low solubility in aqueous media. To reduce hydrophobicity and increase solubility in aqueous media, a water-soluble version of PCB (WSPCB) was synthesized. WSPCB is structurally similar to PCB, but it has a mixed polarity spacer arm between the biotin and 1-(2-nitrophenyl)ethyl moieties (Figure 1A). The synthesis of WSPCB (Figure 1B) involved the production of a mono-biotinylated derivative of 2,2'-(ethylenedioxy)-bis(ethylamine), which was then conjugated to 5-succinylamidomethyl-2-nitroacetophenone. The resulting intermediate was converted to a reactive NHS derivative.

In order to determine if the infectivity of adenoviral vectors could be eliminated by treatment with either

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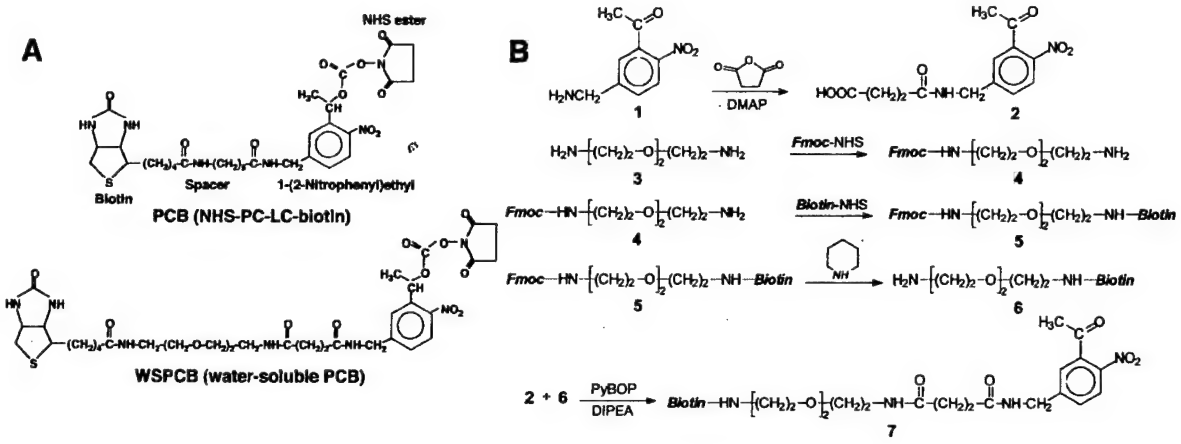

Figure 1. Structures and Synthesis of Photocleavable Biotins

(A) Structures of photocleavable biotin (NHS-PC-LC-Biotin; PCB) and its water-soluble derivative, WSPCB.

(B) Synthesis scheme of WSPCB.

PCB or WSPCB, adenoviral vectors were exposed to various concentrations of PCB or WSPCB. The adenoviral vectors used contain a transducable *lacZ* gene, which provides a simple means of detecting transduced cells. Treatment with WSPCB at 1 mg/ml or greater virtually eliminated the infectivity of these vectors (Figure 2A). In contrast, treatments with PCB were considerably less effective at inhibiting the infectivity of these vectors than with WSPCB at the same concentrations (Figure 2B). Retroviral vectors, when treated with PCB, showed a behavior contrary to this finding [7]. PCB was highly effective at abrogating retroviral infectivity at concentrations of 1–2 mg/ml. This differential sensitivity of retroviral and adenoviral vectors to these two biotinylation reagents may be derived from the vast differences in the structures and properties of the outer surfaces of these viruses. Having observed the sensitivity of the adenoviral vectors to biotinylation by WSPCB, we determined the concentration range in which WSPCB could function to modulate viral infectivity (Figure 2C). Treatment with as low as 0.1 mg/ml WSPCB reduced infectivity by nearly 50%. Treatment with WSPCB at concentrations greater than 0.4 mg/ml virtually eliminated the infectivity of these vectors.

We tested whether the infectivity of the biotinylated adenoviral vector could be recovered via photocleavage of the WSPCB molecule from the viral particles. Adenoviral vectors were treated with various concentrations of WSPCB, and then either kept in the dark or exposed to 365 nm light. These samples were analyzed for their infectivity by using D-17 cells (Figure 3A). WSPCB-treated, nonirradiated adenoviral vectors showed virtually no infectivity. In contrast, when WSPCB-treated viral vectors were exposed to 365 nm light, their infectivity was restored. At an energy output of 16 mW/cm^2, restoration of infectivity occurred within 1 min of exposure to 365 nm light, with maximum recovery occurring after 3 min of irradiation (Figure 3B). Irradiation with 365 nm light beyond 3 min did not enhance the recovery of infectivity. Instead, the infectivity of irradiated vectors decreased slightly with prolonged exposure, possibly because of damage to adenoviral vectors caused by shorter-wavelength radiation emitted from the UV light source.

We next attempted to determine whether the reactivation of adenoviral infectivity could be done in situ in the presence of target cells. Adenoviral vectors treated with 1 mg/ml WSPCB were added to D-17 cells growing in borosilicate glass vials. These vials were either kept in the dark or irradiated with 365 nm light. Infection assays of cells, which had been exposed to WSPCB-treated adenovirus vectors and kept in the dark, showed negligible amounts of infection, while great amounts of infection were observed in cells exposed to WSPCB-treated adenoviral vectors and irradiated with 365 nm light (Figure 4). Irradiation with 365 nm light showed no appreciable effects on cell viability. These experiments were performed by using WSPCB-treated adenoviral vectors, which had been washed of free, unreacted WSPCB by repeated rounds of ultrafiltration. This ensured that the photoactivatable infectivity of adenoviral vectors, shown above, would not be based on the presence of non-virion-associated WSPCB.

The results described above imply that WSPCB is covalently attached to viral particles upon treatment and that the association of WSPCB with virions is responsible for the photoactivatable infectivity of treated vectors. To determine whether WSPCB is associated with virions, and, if so, whether irradiation with 365 nm light causes cleavage of virion-associated WSPCB, Western blotting analysis was performed. Adenoviral vectors were treated with 1 mg/ml WSPCB and either irradiated with 365 nm light or kept in the dark. The resulting viral proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), transferred to a membrane, and probed for conjugated WSPCB with a streptavidin-alkaline phosphatase conjugate (Figure 5). Biotinylation was observed on 84 kDa and 134 kDa proteins in samples derived from WSPCB-treated, nonirradiated adenoviral vectors (lane 1). The amount of WSPCB, bound to these proteins, was markedly reduced when WSPCB-treated viral vectors had been ex-

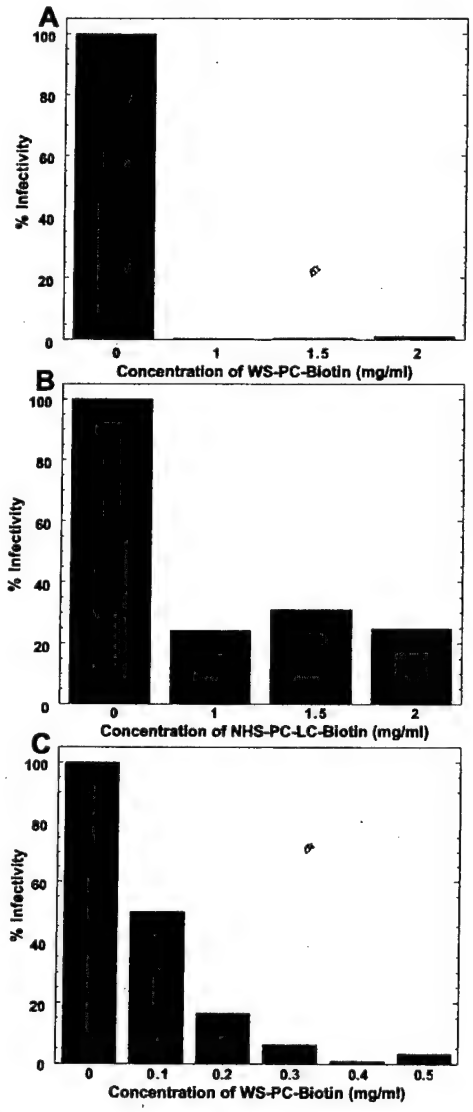

Figure 2. Effect of Treatment with WSPCB or PCB on the Infectivity of Adenoviral Vectors

A stock solution of WSPCB or PCB, both in DMF, was diluted in PBS (pH 7.4). The diluted WSPCB or PCB (25 μl) was allowed to react with adenoviral vectors (2.5×10^8 viral particles in 25 μl PBS) for 2 hr. The infectivity of the resulting viral vectors was assayed by using D-17 cells. DMF at concentrations used during treatment with WSPCB or PCB (up to 8%) showed no effect on infectivity and cell viability. (A and C), WSPCB; (B), PCB.

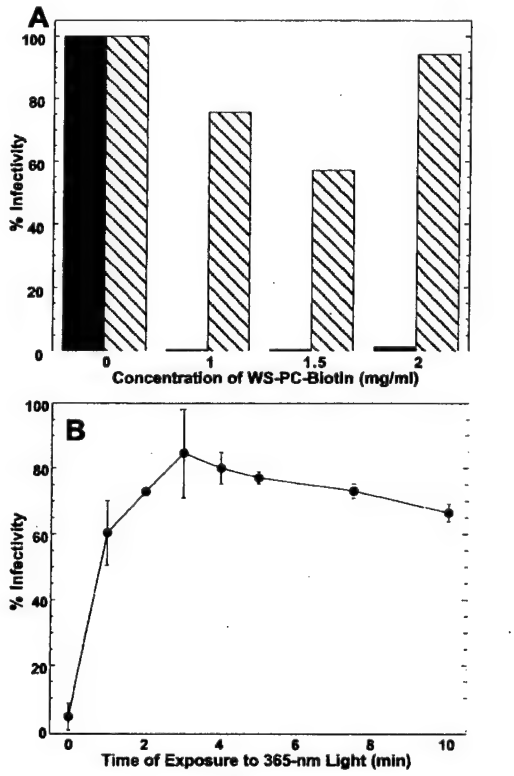

Figure 3. Photoactivation of WSPCB-Treated Adenoviral Vectors

(A) Photoactivation of the infectivity of adenoviral vectors treated with various concentrations of WSPCB. Adenoviral vectors were treated with various concentrations of WSPCB, placed into borosilicate glass vials, and either kept in the dark (solid bars) or irradiated with 365 nm light (16 mW/cm²) for 3 min (hatched bars). The resulting viral vectors were added to monolayers of D-17 cells (5×10^4 per well) to analyze their infectivity.

(B) Time course of the restoration of the infectivity of WSPCB-treated adenoviral vectors. Adenoviral vectors were treated with 1 mg/ml WSPCB and irradiated with 365 nm light (16 mW/cm²) for the durations indicated. The infectivity of the irradiated viral vectors was analyzed by using D-17 cells.

posed to 365 nm light (lane 2). These results demonstrate that irradiation of WSPCB-treated adenoviral vectors with 365 nm light cleaves and subsequently liberates WSPCB from virions. These data strongly suggest that the conjugation of WSPCB to adenoviral vectors and cleavage of virion-associated WSPCB is the infectivity-controlling factor.

As shown above, WSPCB is vastly more effective than PCB in inhibiting the infectivity of adenoviral vectors. The primary difference in properties between the two biotinylation reagents, i.e., hydrophilicity, suggests that the mechanism of infectivity inhibition by these reagents is based on the modulation of the function of viral proteins on the outer, solvent-exposed viral surface. Hence we hypothesized that the ability of adenoviral vectors to either bind or enter target cells is disrupted upon treatment with WSPCB. To test this hypothesis, we devised an assay that quantifies the ability of viral particles to be adsorbed by target cells. Adenoviral vectors were treated with 1 mg/ml WSPCB and incubated over a monolayer of D-17 cells in a culture dish or in an empty culture dish for 3 hr. Then, culture supernatants containing unbound viral vectors were collected, exposed to 365 nm light, and added to fresh D-17 cells for analysis of infectious titers. Comparison of the infectious titers of adenoviral vectors incubated with D-17 cells and those incubated in an empty culture dish indicates a percentage of viral vectors that were associated with the D-17 cells (Figure 6). WSPCB-treated adenoviral vectors exhibited minimal levels of cell association. Adenoviral vectors, which had been treated with WSPCB and irradi-

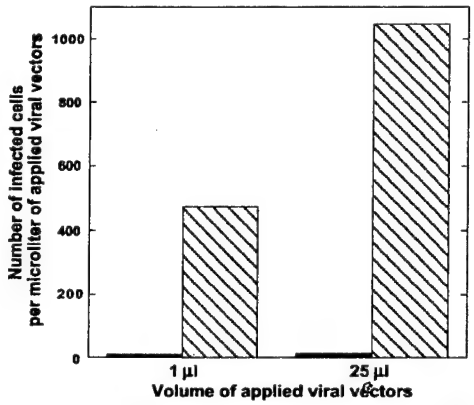

Figure 4. In Situ Photoactivation of WSPCB-Treated Adenoviral Vectors

Adenoviral vectors were treated with 1 mg/ml of WSPCB. The resulting viral vectors were purified away from unreacted WSPCB by ultrafiltration, and small aliquots (1 µl or 25 µl) of the purified viral vectors were placed within each of four 3 ml borosilicate glass vials containing monolayers of D-17 cells (5×10^4 per vial) covered with 500 µl of DMEM/6% FBS. Two of the vials were placed in the dark (solid bars), while the other two vials were exposed to 365 nm light (16 mW/cm^2) for 4 min (hatched bars). At 48 hr after irradiation, cells were stained for the expression of the *lacZ* gene. Shown are the results from two independent experiments with different viral inocula.

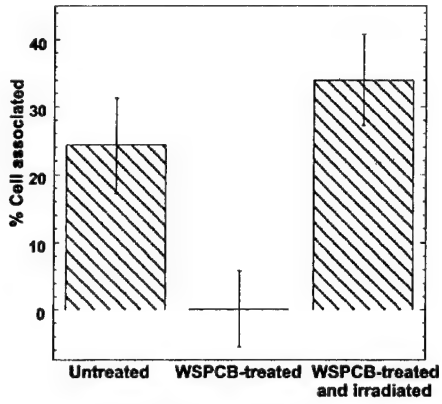

Figure 6. Adsorption Assays of WSPCB-Treated Adenoviral Vectors

Adenoviral vectors were treated with 1 mg/ml WSPCB and incubated for 3 hr over monolayers of D-17 cells grown in a 35 mm culture dish. The same viral vector sample was also incubated in an empty 35 mm culture dish to estimate nonspecific binding of virions to culture dishes. The supernatants (culture medium fractions) containing unbound virions were collected and exposed to 365 nm light (16 mW/cm^2) for 3 min. Adenoviral vectors, which had been treated with 1 mg/ml WSPCB and exposed to 365 nm light prior to the application to D-17 cells or an empty tissue culture dish, were also used in the same manner. Adenoviral vectors without WSPCB treatment were used as controls. All supernatants were subjected to infectivity analysis using fresh D-17 cells. The percentage of cell-associated virions for each sample is calculated as: [(Infectivity remaining in the supernatant after incubation in an empty dish) – (infectivity remaining in the supernatant after incubation with D-17 cells)] / (infectivity remaining in the supernatant after incubation in an empty dish).

ated with 365 nm light prior to incubation with D-17 cells, showed levels of cell association equivalent to those of untreated adenoviral vectors. This data suggests that the conjugation of WSPCB to adenoviral vectors inhibits their infectivity by interfering with their ability to bind to target cells and that irradiation with 365 nm light restores the binding ability of these vectors for target cells by releasing virion-associated WSPCB.

Following the successful development and characterization of photoactivatable adenoviral vectors in an in vitro setting, we tested the potential of these adenoviral vectors to be used for in vivo gene transfer applications using tumors growing in nude mice as viral targets. Athymic nude mice were each injected percutaneously with D-17 cells on each side of the back of the mice just above the upper legs. After the formation of small tumor nodules on both sides of the mice, tumor sites were injected with adenoviral vectors that had been treated with 0.5 mg/ml WSPCB (these treated viral vectors showed photoactivatable infectivity in vitro with cultured D-17 cells; data not shown). The mice were covered in an aluminum foil cloak, which was designed and cut in a way such that only one tumor site was exposed. Exposed tumor sites were irradiated externally with 365 nm light for 4 min through the skin over the tumor nodule. The mice were sacrificed at 48 hr after irradiation, and the tumor nodules, along with adjoining tissue, were collected, sectioned, and stained for the expression of the *lacZ* gene. Sections of tumor sites that were not exposed to 365 nm light show very few, if any, infected cells (Figure 7). However, sections of injected tumor sites that were irradiated with 365 nm light through the skin show a large number of infected cells. This indicates that the infectivity of WSPCB-treated adenoviral vectors can be reactivated in vivo by external photoirradiation. This also demonstrates that the inactivation of viral infectivity with WSPCB is a modification that is not reversed under physiological, unirradiated conditions. Apparently, the skin did not function as a complete barrier to the reactivating light. The skin that was positioned over the irradiated tumor sites was also not noticeably affected by the irradiation. A control

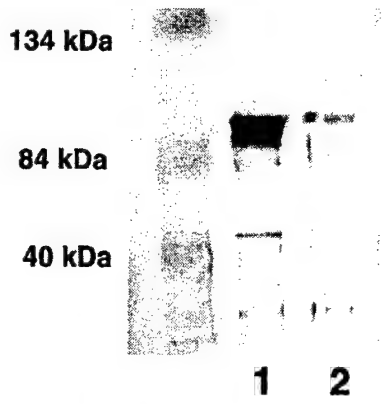

Figure 5. Western Blotting Analysis of WSPCB-Treated Adenoviral Vectors

Adenoviral vectors were treated with 1 mg/ml WSPCB and either kept in the dark (lane 1) or irradiated with 365 nm light (16 mW/cm^2) for 4 min (lane 2). The resulting viral vectors were subjected to SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membrane. Biotinylated viral proteins were detected by using a streptavidin-alkaline phosphatase conjugate (Pierce).

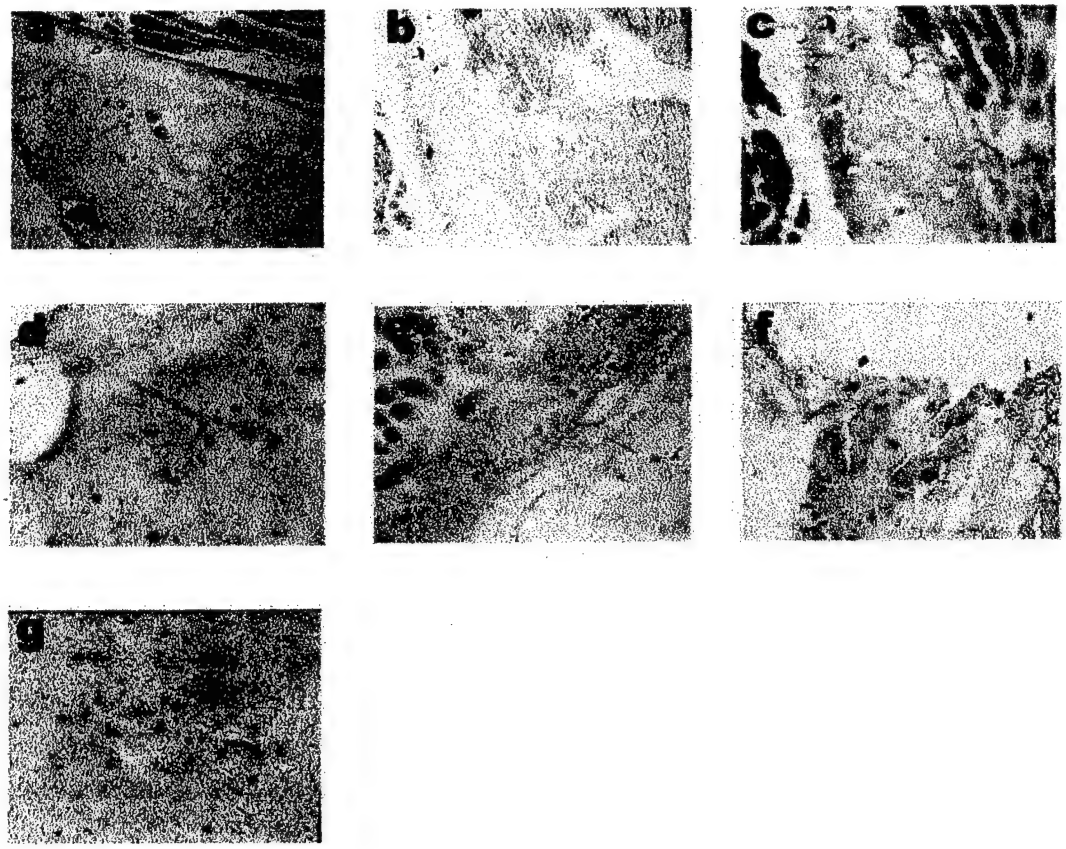

Figure 7. In Vivo Photoactivation of WSPCB-Treated Adenoviral Vectors

Mouse subcutaneous tumor models were prepared by using D-17 cells and athymic nude mice as described in Experimental Procedures. After small tumor nodules (3–5 mm) formed in these mice, each tumor site was injected with WSPCB-treated adenoviral vector (250 μl). Mice were anesthetized and covered with aluminum foil with a hole to expose only the right-hand tumor sites. Exposed tumor sites were irradiated with 365 nm light for two 2 min periods, separated by a 30 s intermission. At 48 hr after irradiation, mice were sacrificed, followed by the collection of tumor sites with adjoining tissue. Tissue was sectioned (50 μm sections), and the resulting tissue sections were stained for the expression of the *lacZ* gene using X-gal as the substrate. The stained tissue sections were viewed under a light microscope. (A–C), no irradiation (control); (D–F), irradiated with 365 nm light. A control experiment was performed in the same manner by injecting unmodified adenoviral vectors into the tumor site (G).

experiment, in which unmodified adenoviral vectors were injected in the same manner, showed a comparable amount of infection, indicating that the reactivation of PC-biotin-modified adenoviral vectors by photoirradiation under in vivo conditions is also highly efficient as seen with in vitro systems. These data demonstrate the potential that WSPCB-treated adenoviral vectors can be used as activatable gene transfer agents in whole animals.

Significance

We have demonstrated a method of making adenoviral vectors selectively activatable by an external stimulus. Conjugation of a PCB derivative, WSPCB, virtually eliminates the infectivity of adenoviral vectors in a reversible manner. Exposure of WSPCB-treated viral vectors to 365 nm light restores infectivity to levels approaching those prior to biotinylation. Inhibition and restoration of the infectivity of adenoviral vectors has successfully been demonstrated both in vitro and in vivo. This infectivity trigger holds considerable potential for the delivery of adenoviral vectors, since the site-specific activation of viral vectors could be mediated by the application of 365 nm light focused on the target sites. The viral surface biotin moiety should also be useful for further modification of the viral surface. For example, biotinylated materials can be attached to viral surface biotin moieties by using streptavidin as a molecular bridge with little effect on the cleavage efficiency of virion-associated PCB by photoirradiation (M.W.P., D.A.H., and T.S., unpublished data). These findings offer a novel strategy to the field of gene therapy by creating a new way to control the activities of viral vectors.

Experimental Procedures

Adenoviral Vectors and Target Cells

The adenoviral vector used in this study, Ad5.CMV-LacZ (Qbiogene, Montreal, Canada), is derived from adenovirus serotype 5 with the deletion of the viral E1A, E1B, and E3 genes. The adenoviral vector carries the bacterial *lacZ* gene (β-galactosidase) under the control

of the human cytomegalovirus immediate-early promoter. This viral vector was produced by using 293A cells (Qbiogene), a subline of 293 cells (human embryonal kidney cells transformed by sheared adenovirus serotype 5 genome), and purified by two rounds of CsCl gradient centrifugation, followed by removal of CsCl by dialysis against 10 mM Tris-Cl (pH 8.0), 2 mM $MgCl_2$, 4% sucrose [8, 9]. The original preparation was diluted to 1×10^{10} viral particles/ml (1×10^9 infectious units/ml) and stored at −70°C until used. The dog osteosarcoma cell line D-17 (ATCC number, CCL-183; American Type Culture Collection) was maintained in DMEM supplemented with 6% FBS (BioWhittaker).

Infectivity Assays

The infectivity of adenoviral vectors was determined by using D-17 cells as targets, which are highly permissive to infection by adenoviral vectors. An adenoviral vector stock was placed over monolayers of 5×10^4 D-17 cells in a 24-well plate and incubated at 37°C for 24 hr. Cells were washed once with culture medium and incubated at 37°C for 24 hr to permit the expression of the *lacZ* gene. Cells were then fixed with 0.5% glutaraldehyde and stained for β-galactosidase activity using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as the substrate. Infected, *lacZ*-expressing cells (stained blue) were counted under a light microscope.

Synthesis of Photocleavable Biotinylation Reagents

A photocleavable biotin (PCB; NHS-PC-LC-biotin) (Figure 1A) was synthesized as previously described [6]. A water-soluble derivative of PCB, WSPCB (Figure 1A), was synthesized by using the following procedure (Figure 1B). All chemicals were obtained from Aldrich except benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), which was from Novabiochem. 5-Aminomethyl-2-nitroacetophenone hydrochloride (1.73 g, 7.5 mmol) (compound 1) was dissolved in 60 ml of dimethylformamide (DMF). To this solution, *N,N'*-diisopropylethylamine (DIPEA; 1.2 ml), 2,6-dimethylaminopyridine (DMAP; 0.46 g, 3.75 mmol), and succinic anhydride (0.75 g, 7.5 mmol) were added. The reaction mixture was stirred at room temperature overnight, added to 120 ml of 0.1 M HCl, and extracted three times, each with 50 ml of chloroform. Organic extracts were combined, dried, and evaporated. Crude products were recrystallized from acetonitrile to give compound 2 (1.2 g, 49% yield). To a stirred solution of 2,2'-(ethylenedioxy)-bis-(ethylamine) (2 g; 13.5 mmol) (compound 3) in 100 ml of acetonitrile, a solution of 9-fluorenylmethoxycarbonyl *N*-hydroxysuccinimide (Fmoc-NHS; 4.95 g, 14.8 mmol) in 50 ml of acetonitrile was added over the course of 30 min. The reaction mixture was stirred for an additional 1 hr, concentrated under reduced pressure, and purified on a silica gel using 0%–6% methanol step-gradient in chloroform/0.8% triethylamine. Fractions containing a mono-Fmoc derivative were pooled and evaporated to give 2.50 g of compound 4 (52% yield). To a stirred solution of compound 4 (2.5 g, 7 mmol) in 50 ml of methanol, a solution of biotin-NHS (2.63 g, 7.7 mmol) in 60 ml of 95% methanol was added over the course of 15 min. After 1 hr at room temperature, thin layer chromatography (chloroform/methanol/acetic acid, 9:1:1 v/v/v) showed complete conversion into compound 5. The mixture was then concentrated under reduced pressure and purified on a silica gel using a 0%–6% methanol step-gradient in chloroform to give 2.2 g of compound 5 (54% yield). Compound 5 (2.2 g, 3.8 mmol) was added to 6 ml of 20% piperidine in DMF. The resulting solution was stirred at room temperature for 10 min, concentrated to about 2 ml under reduced pressure, and added to 20 ml of cold ether. After incubation at −70°C for 30 min, the precipitate was collected by centrifugation. The precipitate (compound 6) was dissolved in 2 ml of methanol, reprecipitated as above, and dried (yield 1.1 g, 82%). Compound 2 (5-succinylamidomethyl-2-nitroacetophenone) (0.59 g, 1.94 mmol) was dissolved in 3 ml of DMF. To this solution, a solution of PyBOP (0.99 g, 1.94 mmol) in 3 ml DMF was added, followed by the addition of *N,N*-diisopropylethylamine (0.68 ml, 3.9 mmol). The resulting solution was stirred at room temperature for 15 min, and then a solution of compound 6 (0.68 g, 1.94 mmol) in 3 ml DMF was added. Stirring continued overnight, and solvents were evaporated under reduced pressure. The residue was purified on a silica gel column using a 0%–20% step-gradient of methanol in chloroform to give 0.75 g of compound 7 (67% yield). Reduction with sodium borohydride and conversion to the target NHS carbonate were carried out as described previously [6].

Treatment of Adenoviral Vectors with PCB and WSPCB

A stock solution of PCB (25 mg/ml in DMF) was diluted in PBS (pH 7.4), and the diluted PCB (25 μl) was added to 2.5×10^8 adenoviral vector particles (2.5×10^5 infectious units) in PBS (25 μl). The biotinylation reactions were performed on ice in the dark for 2 hr and terminated by the addition of 100 μl DMEM/10% FBS. Treatment of adenoviral vectors with WSPCB was performed in the same manner, except that the WSPCB stock solution used had a concentration of 100 mg/ml in DMF.

Photoirradiation of WSPCB-Treated Adenoviral Vectors

Adenoviral vectors were treated with various concentrations of WSPCB as above. WSPCB-treated viral vectors were divided into two groups of borosilicate glass vials. One group of borosilicate glass vials was kept in the dark, while the other group was irradiated for 3 min with 365 nm light using a UV lamp (model B-100 SP, UV Products) equipped with a 160 W mercury vapor bulb, which emits long-wavelength UV light in the 355–375 nm range, peaking at 365 nm. Actual light intensities in this and other experiments were determined by using a UV light meter (model 06-662-65, UV Products). The infectivity of the nonirradiated and irradiated adenoviral vectors was analyzed by using D-17 cells. The time course of the infectivity activation of WSPCB-treated adenoviral vectors upon photoirradiation was investigated in a similar manner, except that the exposure time to 365 nm light was varied.

Western Blotting Analysis

Adenoviral vectors, which had been treated with WSPCB as above, were placed in borosilicate glass vials (1.2×10^9 viral particles per vial). One vial was kept in the dark, while the other vial was exposed for 4 min to 365 nm light as above. Each of these viral samples was centrifuged at 25,000 × g for 2 hr at 4°C to precipitate adenoviral vectors. Viral precipitates were suspended in 10 μl of an SDS sample solution containing 20 mM 2-mercaptoethanol and electrophoresed on 4%–20% polyacrylamide gradient gels [10]. Proteins were transferred from the gel to polyvinylidene difluoride membrane (Millipore) by using a semidry electroblotter. The membrane was blocked with SuperBlock (Pierce) and then incubated for 30 min with a streptavidin-alkaline phosphatase conjugate (Pierce), diluted 5000-fold in SuperBlock. Bound streptavidin-alkaline phosphatase conjugates were visualized by alkaline phosphatase activity using nitroblue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt (Pierce) as the substrates.

Virus Adsorption Assay

Adenoviral vectors (1.25×10^9 viral particles per reaction) were treated with 1 mg/ml WSPCB as above. The resulting viral vectors were either irradiated with 365 nm light (16 mW/cm²) for 3 min or kept in the dark. Each of these samples was added to a monolayer of D-17 cells cultured on a culture dish, and the mixtures were incubated at 37°C for 3 hr. The supernatant (culture medium fraction) containing unbound adenoviral vectors was collected and irradiated with 365 nm light for 3 min as above (this irradiation step was omitted for adenoviral vectors that had been irradiated with 365 nm light). The infectivity of the resulting viral vectors was analyzed by using fresh D-17 cells to estimate the amount of adenoviral vectors that remained unbound. Adenoviral vectors without WSPCB treatment were used as controls. Nonspecific binding of adenoviral vectors was estimated by using empty culture dishes.

In Vivo Activation of WSPCB-Treated Adenoviral Vectors

Mouse subcutaneous tumor models were prepared by using D-17 cells and 9-week-old athymic nude mice (Hsd: Athymic Nude-*nu/nu*; Harlan). D-17 cells (2×10^7 in 200 μl PBS per injection) were injected percutaneously on each side of the back of the mice just above the upper legs. After 24 hr, small tumor nodules (3–5 mm) formed in these mice on both sides (at both injection sites). Adenoviral vectors were treated with 0.5 mg/ml WSPCB as above. In vitro analysis using D-17 cells showed that the infectivity of the resulting WSPCB-

treated viral vectors was inhibited to near completion and that the infectivity can be reactivated efficiently upon irradiation of 365 nm light.

Each tumor site of the mice was injected with 250 μl of the WSPCB-treated adenoviral vectors (2.3×10^9 infectious units prior to the treatment) prepared above. After briefly massaging the injection sites, each mouse was anesthetized and covered with aluminum foil with a hole to expose only the right-hand tumor sites. Exposed tumor sites were irradiated at a distance of 12 cm with a 365 nm UV lamp (model B-100SP) for two 2 min periods, separated by a 30 s intermission. Mice were maintained for 48 hr and then sacrificed, followed by the collection of tumor sites with adjoining tissue. Tissue was sectioned (50 μm sections) in a CM 1850 Cryostat (Leica) at −14°C. Tissue sections were stained for the expression of the *lac*Z gene using X-gal as the substrate, and the stained tissue sections were viewed under a light microscope. A control experiment was performed in the same manner by injecting an equal amount of unmodified adenoviral vectors into a tumor site.

Acknowledgments

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Research News

Gene therapy sees the light

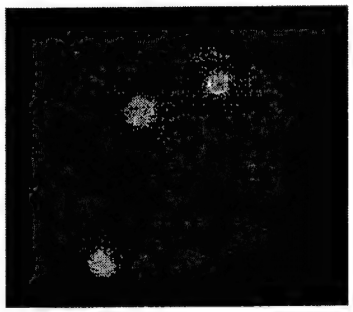
A light-activated vector offers a new strategy for a targeted and inducible gene delivery. Although gene therapy has advanced from proof of principle to early stage clinical trials, it has not proven to be the medical panacea that many had hoped, due to problems associated with efficiency and precision of gene delivery. Viral vectors are the current delivery method of choice, but these friendly Trojan horses can infect a wide range of tissues *in vivo*. In the December issue of *Gene Therapy*, Pandori and Sano report the construction of a photo-activated vector whose infectivity can be controlled temporally and spatially. Attachment of a biotin derivative to an amphotropic murine retrovirus containing the *lacZ* reporter gene inhibited infectivity of the retrovirus vector while in transit. Upon reaching the target site, the biotin molecule was cleaved by exposure to light of wavelengths between 300–365 nm, allowing the vector to infect cells. Thus, infectivity can be inhibited and then activated under controlled conditions, making this vector construct a potentially safer and more efficient gene delivery vehicle.

At last, a ligand for RXR

A long-sought ligand for the orphan retinoid-X receptor (RXR) been identified as a polyunsaturated fatty acid involved in brain development. RXR is a nuclear receptor that heterodimerizes with other proteins, such as the retinoic acid receptor, to function as a ligand-activated transcription factor. Although RXR is activated by the vitamin A metabolite *in vitro*, little is known about ligands that can activate RXR *in vivo*. In the 15 December issue of *Science*, Mata de Urquiza *et al.* report the isolation of an endogenous RXR ligand from mouse brain tissue. Mass spectrometry analysis revealed the ligand to be the polyunsaturated fatty acid DHA. The authors show that DHA specifically binds and activates RXR, but does not activate other similar receptors such as the retinoic acid receptor. DHA is expressed in mammalian brain during early stages of development, and has been shown to be required for brain maturation in rodents and people. DHA-deficient rats and humans develop learning defects, and the fatty acid is also known to influence metabolism and energy homeostasis. The authors suggest that DHA influences neural function through activation of the RXR signaling pathway.

Fetal FISH

Researchers in Hong Kong have developed a non-invasive prenatal diagnostic test for Down syndrome. Fetal chromosomal abnormalities such as trisomy 21, the cause of Down syndrome, are currently detected through invasive procedures such as amniocentesis. In the 25 November issue of *The Lancet*, Poon *et al.* report that intact fetal cells are present in the plasma of pregnant women, and that these cells can be harvested and genetically analyzed. Using fluorescent *in situ* hybridization (FISH), the authors were able to detect the presence (picture) or absence of fetal trisomy 21 in fetal cells isolated from ten maternal plasma samples. These results are surprising, as plasma was believed to be acellular, although fetal DNA has been previously detected in maternal blood. The authors were also able to use the test to determine the sex of the fetuses as early as the end of the first trimester of pregnancy. All results were confirmed by karyotypic analysis of amniotic fluid. Ultimately, with further technical refinements, prenatal diagnosis by maternal plasma DNA analysis may be a safe approach for detecting genetic defects and chromosomal abnormalities.



Power beads

Forget healing crystals—drug-releasing beads may be the next therapeutic delivery method. In the January issue of *Nature Biotechnology*, two studies describe the creation of a protein-producing matrix by mixing genetically-engineered kidney epithelial cells with a copolymer derived from seaweed. This mixture forms a beadlike matrix that allows free exchange of proteins, nutrients and oxygen. Read *et al.* and Joki *et al.* created encapsulated cells that constitutively overexpress endostatin, an anti-angiogeneic protein fragment that is currently being tested in clinical trials as an anti-cancer drug. Read *et al.* report that intracerebral implantation of these cells prevents tumor formation and growth in the brains of immunocompetent rats, while Joki *et al.* report that the implanted capsules reduce the growth of existing tumors by 70%. Studies have shown that continuous administration improves endostatin efficacy in mice, but would require patients to receive frequent injections or carry a delivery apparatus. The encapsulated cells survive and maintain endostatin production for at least four months after intracerebral implantation. They also exclude inflammatory cells, protecting the cells from rejection. These beads may be developed as a simplified method to continuously deliver anti-angiogenics and other therapeutic proteins to people.

Diabetes signals

Two studies published in recent issues of *Nature* shed light on the signal transduction pathways underlying Type II diabetes mellitus. In the 14 December issue, Hart *et al.* report that pancreatic β cells express fibroblast growth factor receptors and ligands. Transgenic mice that produce a mutant form of the FGF receptor FGFR1c developed a phenotype resembling type II diabetes in humans, including fewer β cells, deficits in glucose homeostasis, and impaired insulin processing. The homeobox gene Ipf1 has been genetically linked to diabetes and the authors show that this gene regulates FGFR1c expression. Thus, the FGF pathway may mediate the nutritional and mitogenic control of β-cell expansion and function, and future studies should determine whether aberrant FGF signaling also contributes to human diabetes. In a second study published in the 21/28 December issue, Pende *et al.* show that mice deficient in S6 kinase (S6K1), a member of the PI3-kinase signaling pathway, develop a phenotype resembling malnutrition-induced type II diabetes. S6K1-deficient mice develop hypoinsulinemia and glucose intolerance, due to a decrease in β-cell size and decreased insulin secretion. Thus, S6K is involved in glucose homeostasis, and may underlie the link between early malnutrition and diabetes.

Contributions by Kristine Novak, Karen Birmingham and Bernd Pulverer

studies with a stable isotope of magnesium (Mg-26) have revealed brand-new information about the magnesium-transport characteristics of smaller tissue structures in the kidney. These studies have opened new avenues of research.

Chandra said that numerous areas of cancer research may benefit from the application of this technique. It can be used to verify the anticancer potential of a drug by locating it in the cell and providing a comparison of its accumulation in normal and cancerous cells. The technique reveals changes in very basic cellular chemical composition (potassium, sodium and calcium levels). This allows the deeper study of cytotoxic actions of other anticancer drugs. Chandra added that the isotopic discrimination of the technique can also be used to study the location of molecules inside a cell by using molecules that have been modified to contain isotopic labels. This clears the way for studying the uptake and location of a wide variety of molecules or their metabolites inside a cell.

"The most interesting part of using [secondary ion mass spectrometry] for biological studies is that the technique is revealing a truthful location of elements and isotopes inside the cell. Even now we know so little about fundamental questions such as what is the role of calcium in cell division and why do cancer cells have abnormal calcium signaling. The application of this new technology may prove to be important in understanding these questions." □

Richard Gaughan

Light-activated vector delivers therapeutic genes on target

BOSTON — Gene therapy is a medical technique that offers the possibility of directing the body's own mechanisms to repair or rebuild damaged cellular structures and functions. For example, if healthy heart cells could be triggered to replace old, damaged blood vessels with new ones, the resulting revascularization could provide significant improvement in heart function.

Delivering the genes is the key. Therapeutically useful genes can be delivered using retroviruses, which carry their genetic information in RNA and produce DNA in the cells they infect. The retroviruses can be encapsulated in glycoprotein envelopes, which are sugar-protein molecules. Genetic manipulation of these envelopes can target them biochemically toward specific cell surface structures, but the modifications required are often unstable, ineffective or even detrimental to the efficiency of the viral infection mechanism.

Dr. Mark Pandori and Dr. Takeshi Sano, researchers at Beth Israel Deaconess Medical Center of Harvard Medical School, have developed a strategy for localizing the delivery of gene therapeutic agents. They modify the envelope protein genetically, rather than for a physical reaction, adding light-sensitive infectivity inhibitors that are unlocked by long-wavelength UV radiation. The glycoprotein envelope thus keeps the retrovirus from infecting any cells until it is irradiated and opened.

The scientists surrounded a well-characterized retroviral vector with a modification reagent constructed from a biotin derivative. The vector, derived from amphotropic Moloney murine leukemia virus, carries a bacterial lacZ gene that turns blue when stained with a specific agent. The viral infectivity can then be easily evaluated because the nuclei of stained infected cells will turn blue. The biotin reagents link to components of the retrovirus, primarily those on the viral envelope glycoprotein. When the reagent is attached to the retroviral envelope, it inhibits the infectivity of the virus. But when exposed to UV light of 300 to 365 nm, the biotin derivative cleaves, restoring the conjugate protein to its original, unmodified form. The retrovirus has its infectivity restored.

Modified retroviral vectors were introduced into cultures of D-17, a strain of cancerous dog cells. Infectivity of cultures kept in the dark was extremely low, while those exposed to 365-nm light for at least three minutes exhibited more than 30 times the dark-culture infectivity. "The key to this work," said Sano, director of the Center for Molecular Imaging Diagnosis and Therapy at Beth Israel Deaconess, "is that the infectivity of retroviral vectors is made externally controllable by light."

Additional tests confirmed that the phenomenon of light-mediated infectivity activation involves the inactivation and subsequent reactivation of the function of the viral envelope glycoprotein. For some of those tests, modified retroviral vectors were irradiated prior to introduction into the cell cultures. Infectivity of the modified, but dark, retroviruses was extremely low. The infectivity increased after two to six minutes of irradiation but decreased when irradiation continued beyond eight minutes. This may be because of additional damage to the virus from shorter-wavelength UV light present in the lamp.

The treatment conditions developed for efficient infection of the D-17 cell line may not be appropriate for all cell lines. Although a number of human cell lines behave similarly, the levels of inactivation varied from line to line, suggesting that conditions may need to be tailored for specific cellular targets.

Pandori and Sano acknowledge that other difficulties lie ahead. For example, for in vivo applications, Sano said, "the challenge would be whether one can manipulate light beams three-dimensionally with sufficient intensities at target sites in the body. If this could be done, our system would be enormously useful for a variety of in vivo gene transfer applications where the timing and/or locations of the delivery of transgenes must be controlled precisely."

Although the technique already provides a valuable research tool, development is continuing. "For example," he said, "our system could be used if one wants to control the timing and/or location of gene transduction for in vitro systems or in experimental animals. ... First, we are refining this strategy. Second, we are applying this strategy to other viral systems, such as those derived from adenovirus, adeno-associated virus and herpes simplex virus. Third, we are developing in vivo gene transfer applications, such as gene therapy and tissue engineering."

Pandori and Sano believe that this is one of the few examples where the fundamental functions of complex biological assemblies could be controlled or switched by external signals. □

Richard Gaughan

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Adenovirus–Microbead Conjugates Possess Enhanced Infectivity: A New Strategy for Localized Gene Delivery

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We have created a novel method for coupling adenoviral vectors to solid microbeads in a way that does not adversely affect the infectivity of the attached virions. This method utilizes the extremely tight interaction between the protein streptavidin and its ligand biotin as a means of tethering viral particles to microbeads. The adenovirus–microbead conjugates that were created functioned as fully infectious entities and possessed several functional advantages over free, unmodified viral particles. The adenovirus–microbead conjugates possessed enhanced ability to transduce target cells in culture. For target cells of a highly permissive nature, this increase in infectivity was modest. However for target cells of moderate to low permissivity, the enhancement of transduction efficiency was substantial. Adenoviral vectors, previously incapable of infecting a particular colon cancer cell line, were made fully infectious on the same cell line when delivered as solid-phase conjugates. Additionally, solid-phase adenovirus–microbead conjugates showed highly limited diffusion in solution, allowing for focused delivery of viral vectors only to cells that come into contact with the conjugates. When the solid phase to which the viral particles were attached had paramagnetic properties, the location of viral infections was tightly controllable by magnetic force through the use of strategically placed magnets. © 2002 Elsevier Science (USA)

Key Words: viral vectors; adenovirus; solid surface; microbeads; paramagnetic microparticles; biotin; (strept)avidin; localized gene delivery.

INTRODUCTION

A major obstacle in the use of viral vectors as therapeutic tools is the inability to control where such vectors are targeted physiologically. Genetic and chemical modifications of viral envelopes or viral surface proteins to contain binding reagents for particular cell types have been very difficult, resulting in limited success (for recent reviews, see Bilbao *et al.,* 1998; Curiel, 1999; Raynolds and Curiel, 1999; Cannon and Anderson, 2000; Hackett and Crystal, 2000; Vile *et al.,* 2000; Monahan and Samulski, 2000; Wolfe *et al.,* 2000; Kay *et al.,* 2001; Ponnazhagan *et al.,* 2001). For cases where the desired sites of genetic transduction are known, it has been possible to target viral vectors simply by focused administration. However, viral vectors administered to particular physiological locations often diffuse to tissues and organ systems distant from their original placement (Huard *et al.,* 1995; Wirtz *et al.,* 1998; Fechner *et al.,* 1999; Barbara *et al.,* 1999; Hackett and Crystal, 2000; Schellingerhout *et al.,* 2000; Gelse *et al.,* 2001). This, combined with the fact that viral vectors used in gene therapy protocols are highly infectious and have very broad tropism, has generated genuine safety issues for gene therapy.

Creative methods to address these safety concerns have not been forthcoming. Viral vectors with "activatable" infectivity are one possible method for controlling the location and timing of viral infections. This has been accomplished in one case by the chemical conjugation of photocleavable infectivity inhibitors to viral vectors, using light as the infectivity-activating agent (Pandori and Sano, 2000; Pandori *et al.,* 2002). While the precise control of the location of viral infections is potentially achievable, the use of light at relatively short wavelengths, needed for efficient activation of viral infectivity, limits the application of such viral vectors to cases where the desired locations of transduction are highly accessible.

Here, we describe a novel strategy for controlling the delivery and location of viral infections. We have attached adenoviral vectors directly and stably onto the outer surface of microbeads. Viral vectors that are attached to these microbeads were shown to possess multiple functional enhancements over free viral vectors in solution. They possess greater infectivity, particularly on poorly permissive cells. They do not diffuse from the areas in which the virus–microbead conjugates are initially placed; and they are highly manipulatable in microbead-conjugated form. Also shown herein is the use of paramagnetic microparticle–adenovirus conjugates

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as gene delivery vehicles whose ability to transduce cells can be controlled spatially by the use of magnetic force.

RESULTS

We have investigated the possibility of using solid microbeads as delivery devices for adenoviral vectors. We hypothesized that, by using the microbeads as virus carriers, the diffusion of such viral particles could be limited, forcing them to infect only those cells or tissue that come in direct contact with the virus–microbead complexes. An essential requirement for this scheme is that such viral vectors retain biological activity (infectivity) while attached to the surfaces of microbeads. We decided to utilize the biotin–(strept)avidin interaction as a means of tethering viral particles to the surfaces of microbeads. Avidin and streptavidin are proteins that exhibit extremely high binding affinities for their ligand biotin ($K_d \sim 10^{-14}$–10^{-15} M) (Green, 1970, 1990; Wilchek and Bayer, 1999). Earlier work performed in our laboratory revealed that chemical, covalent conjugation of biotin moieties (biotinylation) to the outer surfaces of adenoviral vectors can be performed with a very limited effect on the viral infectivity. Hence, such biotinylated viral particles could be stably attached to the surfaces of microbeads that are coated with avidin or streptavidin. A wide variety of microbeads are available that possess avidin or streptavidin covalently attached to their outer surface. In this work, we chose to utilize streptavidin-coated silica microbeads (Bangs Labs, Fisher, IN). These silica microbeads have a density (specific gravity) of 1.95 g/ml, approximately twice that of water, hence strongly limiting their diffusion in solution. Streptavidin-coated microbeads possess a high-binding capacity for biotinylated macromolecules, therefore allowing a strong possibility that biotinylated viral particles could be immobilized stably and tightly on their surface.

To ensure attachment of functional adenoviral vectors to the surfaces of streptavidin-coated microbeads, we determined conditions that allow for biotinylation of adenoviral particles with minimal disturbance of their infectivity. We used sulfo-NHS-LC-biotin (Pierce Chemical, Rockford, IL) as the biotinylation reagent, as it is water-soluble, is easy to use, and possesses a long spacer chain that allows for greater accessibility of the biotin moiety by streptavidin on the microbead surfaces. Additionally, the ability of this biotinylation reagent to react specifically with primary amino groups via its *N*-hydroxy-succinimidyl (NHS) group should lend itself well to the modification of adenoviral surfaces, which consist almost entirely of protein. By testing a range of concentrations of sulfo-NHS-LC-biotin, we found that biotinylation of adenoviral vectors (Ad5.CMV-*lac*Z, which carries the transducable *lac*Z gene) can be achieved with no disturbance of viral infectivity at a treatment concentration of

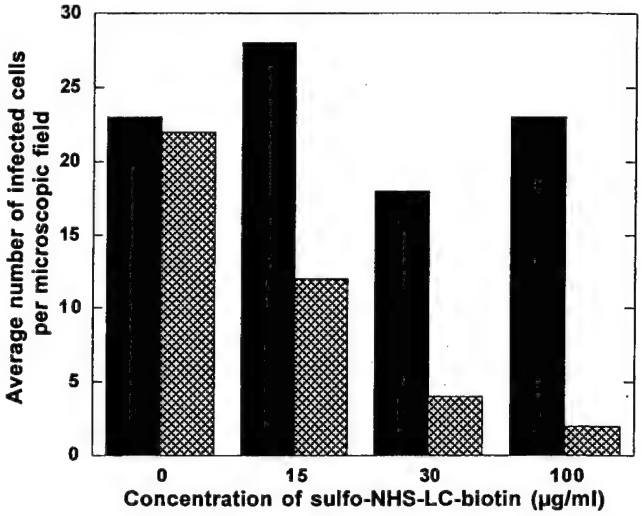

FIG. 1. Infectivity analysis of adenoviral vectors treated with sulfo-NHS-LC-biotin. Adenoviral vectors were treated with varying concentrations (0–100 μg/ml) of sulfo-NHS-LC-biotin for 2 h on ice in the dark, followed by the addition of excess glycine to absorb unreacted sulfo-NHS-LC-biotin. Treated viral vectors were diluted 100-fold in PBS and placed on monolayers of D-17P4 cells. After 48 h of exposure to viral vectors, cells were stained for the *lac*Z expression using X-gal as the substrate (solid bars). Diluted viral vectors were also mixed with excess Neutralite avidin, and the mixtures were titrated on D-17P4 cells in the same manner as above (hatched bars). Each datum shown is the average number of infected cells per microscopic field (2.27 mm^2) and is representative of two independent experiments.

15 μg/ml, in phosphate-buffered saline (PBS) (pH 7.6) (Fig. 1). At this concentration, adenoviral vectors retained 100% of their original infectivity. Adenoviral vectors, treated with sulfo-NHS-LC-biotin, were also mixed with excess Neutralite avidin (a neutralized, deglycosylated form of avidin; Southern Biotechnology Associates, Birmingham, AL) to determine the effect of the conjugation of avidin to the surface of the viral particles on viral infectivity. For viruses that had been treated with sulfo-NHS-LC-biotin, the addition of Neutralite avidin had a negative effect on viral infectivity. The strength of this negative effect correlated with the concentration of sulfo-NHS-LC-biotin used during biotinylation of viral vectors (Fig. 1). This indicates that the surfaces of the adenoviral particles were indeed biotinylated and that biotin moieties on the viral outer surface were accessible by avidin molecules. In addition, adenoviral vectors, treated with 15 μg/ml sulfo-NHS-LC-biotin, were completely removable from solution (capturable) by using an excess amount of streptavidin-coated microbeads (data not shown). This result confirms the biotinylation of viral particles on the viral outer surface and the accessibility of biotin moieties to avidin and streptavidin.

We next attached the biotinylated adenoviral vectors to the surfaces of streptavidin-coated silica microbeads to determine if these viral vectors retained their ability to infect cells while immobilized on a solid support. We investigated two sizes of microbeads, 2.2 and 0.58 μm in

TABLE 1

Infectivity of Adenovirus–Microbead Conjugates on an Engineered Dog Osteosarcoma Cell Line, D-17P4, and a Rat Glioma Cell Line, C6, Compared to Free, Unmodified Adenoviral Vectors

Cell line	Microbead diameter (μm)	Ratio of viral particle to microbead	Viral particles/infectious unit	Microbeads/infectious unit
D-17P4	2.2	3	24 ± 1.6	8.3 ± 0.7
D-17P4	2.2	30	26 ± 0.65	0.88 ± 0
D-17P4	2.2	120	48 ± 3.5	0.40 ± 0
D-17P4	0.58	0.6	31 ± 8	25 ± 12
D-17P4	0.58	6	36 ± 3	4 ± 2
D-17P4	0.58	24	42 ± 11	1.3 ± 0
D-17P4	Free virus	—	61 ± 22	—
C6	2.2	3	95 ± 14	32 ± 4.5
C6	2.2	30	191 ± 76	6 ± 2.6
C6	2.2	120	209 ± 20	1.7 ± 0.2
C6	0.58	0.6	77 ± 26	127 ± 42
C6	0.58	6	80 ± 29	13 ± 5
C6	0.58	24	121 ± 13	5 ± 0.5
C6	Free virus	—	1577 ± 506	—

Note. The data shown are the average of three independent experiments with the standard deviation.

diameter, to determine if the diameter of the microbeads affects the ability of viral particles to function. Adenoviral vectors were biotinylated with sulfo-NHS-LC-biotin, as described above, and washed by repeated ultrafiltration to remove non-virion-associated biotinylation reagent. Biotinylated viral vectors were combined with either 2.2- or 0.58-μm-diameter streptavidin-coated silica microbeads at varying ratios of viral particle to microbead. Analysis of the supernatants of these mixtures after centrifugation revealed no infectivity, indicating that all of the viral particles had been bound to the surface of streptavidin-coated microbeads. The resulting adenovirus–microbead conjugates were washed by repeated centrifugation and resuspension in fresh PBS. Adenovirus–microbead conjugates were then placed over both D-17P4 and C6 cells to evaluate their ability to transduce these cells. D-17P4 cells were chosen because of their high permissivity to adenoviral infection, while C6 cells have demonstratively lower permissivity to such infection. Adenovirus–microbead conjugates were compared to free, unmodified adenoviral vectors for the ability to transduce target cells in culture. As shown in Table 1, adenovirus–microbead conjugates showed ability to infect both D-17P4 and C6 cells. Interestingly, when the efficiency of infection is evaluated in the terms of how many viral particles are required to establish an infectious unit, adenovirus–microbead conjugates were more infectious than unmodified adenoviral vectors present free in solution. For D-17P4 cells, this increase in infection efficiency appears to be modest (up to approximately threefold). However for the lesser permissive C6 cell line, this increase in infection efficiency was dramatic, as approximately 20-fold fewer viral particles were required when adenoviral vectors were delivered by microbeads, as compared to the same viral vectors used free in solution. As the ratio of viral particle to microbead was increased, the infection efficiency was not markedly affected, indicating that the placement of multiple viral particles per microbead might be unnecessary for maximal transduction capability.

Not surprisingly, when the microbeads carrying adenoviral particles are viewed as infectious entities, the infectivity of the microbeads increased notably as the ratio of viral particle to microbead increased. At the highest ratios of viral particle to microbead tested, the infectivity of adenovirus–microbead conjugates reached the point where each microbead can lead to the guaranteed infection of one cell. Certain conditions allowed for even fewer than one microbead to be capable of infecting a target cell (30 or 120 viral particles per microbead for D-17P4; Table 1). This result is derived from the fact that the target cells were rapidly dividing throughout the infection process. When a dividing cell (or a cell primed for division) was infected, it resulted in two infected cells, leading to an overestimation of the number of infection events.

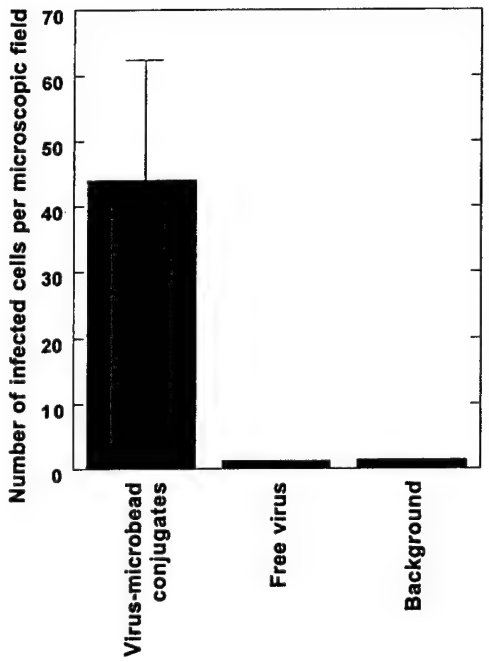

FIG. 2. Infectivity of adenovirus–microbead conjugates on a human colon adenocarcinoma cell line, COLO 205, compared to free, unmodified adenoviral vectors. Adenoviral vectors were treated with 15 μg/ml sulfo-NHS-LC-biotin, washed of non-virion-associated biotinylation reagent by repeated ultrafiltration, and mixed with streptavidin-coated silica microbeads (0.58-μm diameter) at a ratio of 30 viral particles per microbead. The adenovirus–microbead conjugates and free, unmodified adenoviral vectors were titrated for their infectivities on monolayers of COLO 205 cells. At 48 h postexposure to viral vectors, cells were stained for the *lac*Z expression using X-gal as the substrate. COLO 205 cells that were not exposed to adenoviral vectors but treated with X-gal showed a small number of background positive (blue) cells. Each datum shown is the average number of infected cells per microscopic field (2.27 mm^2) with the standard deviation from two independent experiments.

Having evaluated the capability of adenovirus–microbead conjugates to mediate infection of target cells with high to moderate permissivity, we investigated whether the placement of adenoviral vectors on microbeads might allow for the infection of cells that are nonpermissive to adenoviral infection. Based both on previous studies and on our own findings, we chose to evaluate the ability of adenovirus–microbead conjugates to infect COLO 205 cells, a human colon adenocarcinoma cell line. COLO 205 cells are markedly nonpermissive to adenoviral infection, presumably due to the presence of a very limited number of the receptor for adenovirus (coxsackie-adenovirus receptor) (Fechner *et al.*, 2000). We compared the ability of biotinylated adenoviral vectors, bound to 0.58-μm-diameter streptavidin-coated microbeads at a ratio of approximately 30 viral particles per microbead, to infect COLO 205 cells to that of free, unmodified viral vectors. An equal number of viral particles, either in microbead-associated or in free form, was applied to COLO 205 cells to directly compare the efficiency of infection. As shown in Fig. 2, free adenoviral vectors showed very little ability to transduce COLO 205 cells (lower than 8×10^4 infectious units per ml), in agreement with previous studies (Fechner *et al.*, 2000). In contrast, adenoviral vectors that were bound to streptavidin-coated silica microbeads showed marked infection of these cells, possessing a titer of at least 3×10^6 infectious units per ml when used at the same virus concentration as free adenoviral vectors. These data indicate that adenoviral vectors gain substantial advantages over free viral vectors with regard to the ability to infect poorly permissive cells by their placement on solid microbeads. This might be derived from the fact that the density of the adenovirus–microbead conjugates allows for concentration of the viral particles onto the surface of target cells, where their diffusion is highly limited. At this location, the viral particles are forced into proximity of the surfaces of target cells, where the binding of viral vectors to their receptors might occur efficiently even in the presence of a very limited number of the receptor due to the close proximity of viral particles to the receptors.

We next tested the ability of adenovirus–microbead conjugates to function as a tool to direct viral transduction to a specific location. Realizing that the density of adenovirus–microbead conjugates would strongly limit the diffusion of viral vectors in solution, we reasoned that these conjugates could be used to force the localized infection of target cells. To test this, adenovirus–microbead conjugates were made with either 2.2- or 0.58-μm-diameter streptavidin-coated microbeads at a ratio of one or three viral particles per microbead, respectively. These conjugates were then placed into culture dishes containing monolayers of D-17P4 cells. This process included the crafting of specific patterns in each culture dish (the letters M and K). After 48 h, cells were fixed and stained for the expression of the *lac*Z gene. As shown in Figs. 3A and 3B, profound infection of D-17P4 cells can be seen distinctly in patterns in which the adenovirus–microbead conjugates were initially placed. Microscopic examination revealed that infected cells were rarely, if ever, seen in other areas of the cell monolayer not exposed to layered microbeads (data not shown). Clearly, the limited diffusion of the viral particles when they were attached to solid microbeads allowed for a focused, patterned transduction of cells.

Having controlled the location of viral transduction of target cells using adenovirus–microbead conjugates, we hypothesized that the same technique could also be carried out using paramagnetic microparticles as virus carriers. We reasoned that in using paramagnetic microparticles as virus carriers, one could control the location of viral infections through magnetic force by the placement of magnets in areas of desired transduction. To test this, we combined adenoviral vectors, which had been biotinylated with 15 μg/ml sulfo-NHS-LC-biotin, with streptavidin-coated paramagnetic microparticles (Promega). These paramagnetic microparticles are not uniform in size and

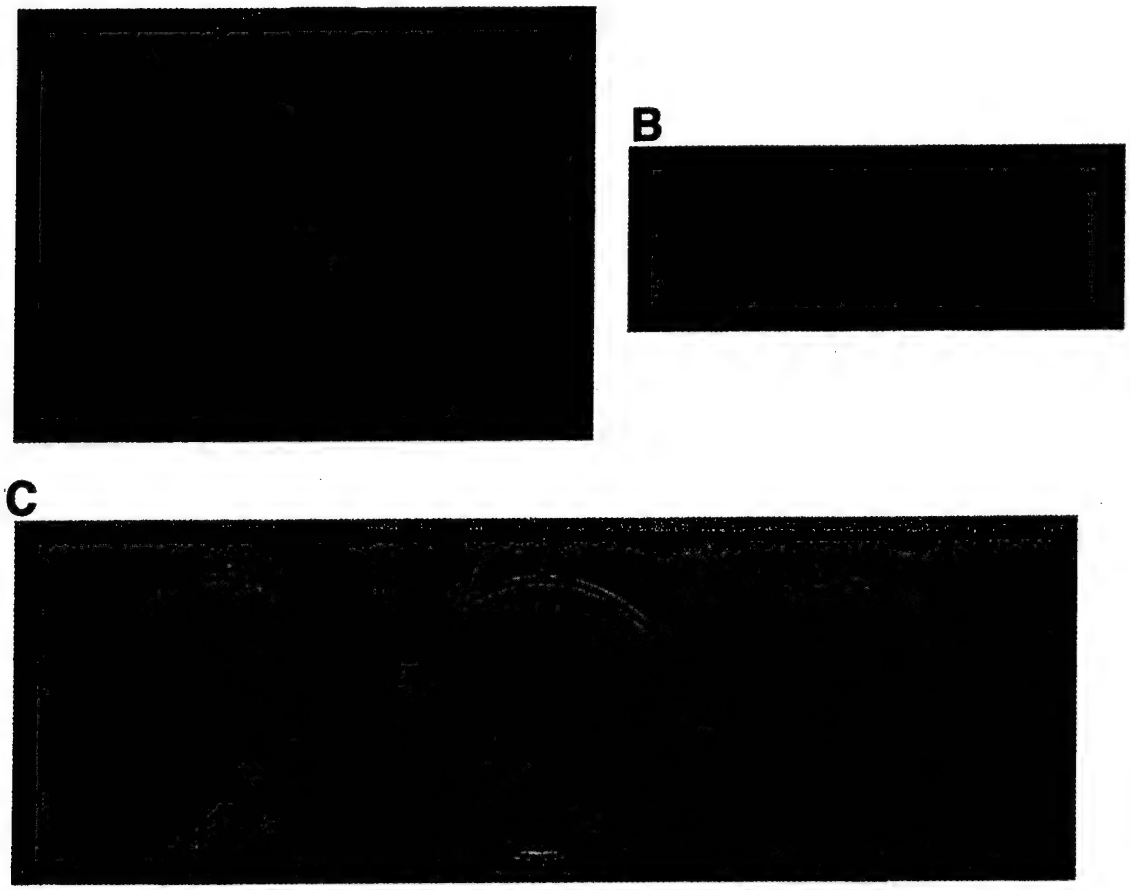

FIG. 3. The use of microbeads or paramagnetic microparticles as adenoviral vector delivery vehicles for localized transduction of target cells. (A) Adenoviral vectors were treated with sulfo-NHS-LC-biotin (30 μg/ml), washed, and mixed with streptavidin-coated silica microbeads (2.2 μm in diameter) at a ratio of one viral particle per microbead. The resulting adenovirus–microbead conjugate suspension (100 μl) was placed over a monolayer of D-17P4 cells in the pattern of the letter "M." At 48 h after the administration of the adenovirus–microbead conjugates, cells were stained for the *lac*Z expression using X-gal as the substrate. (B) Adenoviral vectors, biotinylated as described above, were mixed with streptavidin-coated silica microbeads (0.58 μm in diameter) at a ratio of three viral particles per microbead. A suspension containing these conjugates in PBS (24 μl) was placed over monolayers of D-17P4 cells in the pattern of the letter "K" (left dish). An equal number of free, unmodified adenoviral vectors in the same volume of PBS was also placed over a monolayer of D-17P4 cells in the pattern of the letter "K" (right dish). At 48 h after the administration of the adenovirus–microbead conjugates, cells were stained for the *lac*Z expression as above. (C) Adenoviral vectors were treated with 15 μg/ml sulfo-NHS-LC-biotin and attached to the surface of streptavidin-coated paramagnetic microparticles at a ratio of approximately 600 viral particles per magnetic microparticle). A suspension (2 μl per well) containing paramagnetic microparticle–adenovirus conjugates was placed in three wells of a six-well plate (35-mm well diameter) containing monolayers of D-17P4 cells. Small (5 mm in diameter), round, rare-earth magnets had been taped to the bottom of two of the wells (one magnet placed in the center for the left well; three magnets placed in a diagonal pattern for the center well), prior to the administration of adenovirus–microparticle conjugates. No magnet was affixed to the right well. The six-well plate was then placed at room temperature with gentle shaking for 30 min, and the magnets were then removed from the plate. Cells were cultured for 48 h and then stained for the *lac*Z expression as above.

shape, but they possess an average diameter of 1.0 μm. An equal amount of the paramagnetic microparticle–adenovirus conjugates was placed into each of three 35-mm wells containing monolayers of D-17P4 cells. Small (5 mm in diameter), round, rare-earth magnets had been affixed onto the bottom of two of the wells in either a singular dot pattern or a diagonal pattern of three dots, prior to the application of paramagnetic microparticle–adenovirus conjugates. In the third well, paramagnetic microparticle–adenovirus conjugates were added, but no magnets were affixed to it. These wells were subject to gentle shaking at room temperature for 30 min, at which time the magnets were removed, and cells were cultured for 48 h. The monolayers of cells in the wells were fixed and stained for the expression of the *lac*Z gene. As shown in Fig. 3C, cells were clearly infected in a manner specified by the presence of magnets. Well-defined round spots showing highly infected cells were present in wells where magnets had been affixed prior to the administration of paramagnetic microparticle–adenovirus conjugates. For the well where no magnets were affixed, a random dispersal of infected cells is evident. These results demonstrate that adenoviral vectors, attached to paramagnetic microparticles, retain their bio-

logical activity (infectivity), in a manner similar to adenovirus–microbead conjugates described above, and that these conjugates can be targeted specifically to chosen regions of target cells by using magnetic force for spatially controlled transduction of cells.

DISCUSSION

We have shown that adenoviral particles can be conjugated stably to the surfaces of solid microbeads by using the biotin–(strept)avidin interaction, with no resulting negative effects on the infectivity of the attached virions. Such solid-phase-conjugated adenoviral vectors have been shown to possess certain functional advantages over the equivalent viruses that are free in solution. Conjugation of adenoviruses to silica microbeads results in the creation of viral vectors that have a density (specific gravity) nearly twice that of water, allowing for such virus–microbead conjugates to resist the forces of diffusion present in solutions. Hence, such virus–microbead conjugates may have a role as safer gene therapeutic agents, particularly for situations where it is highly undesirable for applied viral agents to spread into the blood or surrounding tissues, leading to uncontrolled transduction of nontarget tissues. The density of such virus–microbead conjugates can also be controlled, as needed, by using microbeads with appropriate densities as virus carriers.

Another distinct advantage of placing adenoviral vectors on solid microbeads is a marked increase in transduction efficiency for target cells. When compared to free viral vectors, adenovirus–microbead conjugates possessed higher infectivity for a variety of cell lines. In particular, the transduction enhancements gained by solid-phase delivery were demonstrably greater on cells of moderate or poor permissivity. The mechanism(s) of enhanced transduction efficiency is yet to be determined. It seems relevant that the placement of viral particles on the surfaces of microbeads that sink in culture media or physiological solutions due to their high densities would allow viral particles to be concentrated onto the surfaces of cells. The microbeads might then function as an anchor to hold the viral particles in close proximity to the cell surfaces where encounters with necessary viral receptors might occur more readily, since the viral particles cannot lose their proximity to the cell surface and their local concentration on the cell surface is increased. It is possible that their enhanced transduction efficiency might also involve endocytosis of virus–microbead conjugates that is independent of the binding of viral particles to the primary receptor for adenovirus (coxsackie-adenovirus receptor). The endocytosis of a microbead carrying multiple viral particles might occur readily on the surface of a cell, even in the presence of very limited amounts of necessary viral receptors, since microbeads are often rapidly taken up by cells on which they are placed. In other experiments in our laboratory, adenoviral vectors, which were placed on the surfaces of flat microtiter wells using the same streptavidin–biotin chemistry, did not exhibit infectivity enhancements comparable to those of microbead-associated adenoviral vectors, when target cells were placed directly over such adenovirus-coated surfaces (D. A. Hobson, M. W. Pandori, and T. Sano, unpublished data). These differences suggest that the endocytosis of virus–microbead conjugates contributes, at least partly, to the enhanced transduction efficiency seen with adenovirus–microbead conjugates.

Successful attachment of adenoviral vectors to microbeads using the streptavidin–biotin interaction also led us to the creation of similar conjugates using streptavidin-coated paramagnetic microparticles as virus carriers. Adenovirus–paramagnetic microparticle conjugates were highly infectious, similar to adenovirus–microbead conjugates, but they were also highly localizable by utilization of magnetic force. This offers yet another potential method of controlling, externally, the location of virus infections even in complex biological systems.

The generation of virus–microbead/microparticle conjugates may offer other advantages not investigated in this work. One obvious possibility is the ability to attach viral vectors containing different genes of interest onto the same microbeads. This might result in the creation of microbeads that have the ability to guarantee the delivery of multiple genes to the same target cells.

MATERIALS AND METHODS

Virus production and cells

The adenoviral vector used in this study, Ad5.CMV-*lac*Z (Qbiogene, Montreal, Canada), is derived from adenovirus serotype 5 with the deletion of the viral E1A, E1B, and E3 genes. The adenoviral vector carries the bacterial *lac*Z gene (β-galactosidase) under the control of the human cytomegalovirus (CMV) immediate-early promoter with a polyadenylation site. This viral vector was produced by using 293A cells (Qbiogene), a subline of 293 cells (human embryonal kidney cells transformed by sheared adenovirus serotype 5 genome) and purified by two rounds of CsCl gradient centrifugation, followed by removal of CsCl by dialysis against 10 mM Tris–Cl pH 8.0, 2 mM $MgCl_2$, 4% sucrose (Mittereder *et al.*, 1996; Nyberg-Hoffman and Aguilar-Cordova, 1999). The original preparation at a concentration of 1.0×10^{13} viral particles/ml (1.0×10^{12} infectious units/ml) was diluted in PBS to 1.0×10^{10} viral particles/ml (1.0×10^{9} infectious units/ml) and stored frozen at −70°C until used.

The following three cell lines were used as targets for infection by adenoviral vectors: D-17P4 (engineered dog osteosarcoma cells), C6 (rat glioma cells; American Type Culture Collection, ATCC), and COLO 205 (human colon

adenocarcinoma cells; ATCC). D-17P4 is a derivative of the dog osteosarcoma cell line D-17 (ATCC), which has been stably transfected with the plasmid pPSMA2 (Carter *et al.,* 1996; Luthi-Carter *et al.,* 1998) (a gift from Dr. Joseph T. Coyle, McLean Hospital, Belmont, MA). pPSMA2 carries a cDNA for the human prostate-specific membrane antigen (PSMA) under the control of the CMV immediate-early promoter. D-17P4 cells have been shown to express high levels of PSMA on their surface by fluorescence-activated cell-sorting analysis using a monoclonal antibody against PSMA (clone Y-PSMA1; Yes Biotech Lab, Ontario, Canada) (data not shown). D-17P4 and C6 cells were maintained in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 6% fetal bovine serum (FBS; BioWhittaker). COLO 205 cells were maintained in RPMI 1640 (BioWhittaker) supplemented with 10% FBS, 4.5 mg/ml glucose, 1.5 mg/ml sodium bicarbonate, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid).

Biotinylation of adenoviral vectors

Sulfo-NHS-LC-biotin (Pierce Chemical) was used as the biotinylation reagent. Dilutions of a sulfo-NHS-LC-biotin stock solution (3 mg/ml in dimethylformamide) were added to 200 μl (2.4×10^9 viral particles) of an adenoviral vector solution in PBS (pH 7.6) to various final concentrations (0–100 μg/ml). The mixtures were placed on ice, in the dark, for 2 h, and then 90 mM glycine in PBS (pH 7.6) was added to each reaction mixture to absorb unreacted sulfo-NHS-LC-biotin. Three rounds of ultrafiltration using ZM-500 centrifugal filtration units (molecular mass cutoff, 500 kDa; Millipore) with PBS (pH 7.4) containing 0.05% Tween 20 (PBST) were used for the removal of non-virion-associated biotinylation reagent.

The following two assays were performed for initial assessment of the biotinylation of adenoviral vectors. In the first assay method, biotinylated adenoviral vectors, after the addition of 90 mM glycine, were diluted 100-fold in PBS. Aliquots (100 μl) of each reaction mixture were mixed with 8 μl Neutralite avidin (5 mg/ml; Southern Biotechnology Associates) or PBS as a control. The resulting mixtures were incubated for 30 min at room temperature, and their infectivity was analyzed by the following procedure. D-17P4 cells (5×10^4) were seeded in wells (15.5 mm in diameter) of a 24-well plate 24 h prior to infection. Biotinylated adenoviral vectors, with the addition of excess Neutralite avidin or PBS, were placed over monolayers of cells and incubated at 37°C. At 48 h postexposure, cells were fixed with 0.5% glutaraldehyde and stained for β-galactosidase (*LacZ*) activity using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as the substrate. Infected, *lacZ*-expressing cells were readily identifiable by their blue cytoplasm and were counted using a light microscope. Untreated adenoviral vectors were used as controls, in which no effect of the addition of Neutralite avidin was seen (data not shown).

In the second assay method, biotinylated adenoviral vectors (approximately 6×10^8 viral particles in 50 μl) were mixed with streptavidin-coated silica microbeads with a diameter of 2.2 μm (10 μl containing approximately 9.8×10^7 microbeads; Bangs Labs). The mixtures were centrifuged for 3 min at 5000 *g,* and the supernatants were analyzed for the presence of viral particles by infectivity assays using D-17P4 cells as in the first assay method above.

Preparation and analysis of adenovirus–microbead conjugates

Adenoviral vectors were biotinylated with 15 μg/ml sulfo-NHS-LC biotin as described above. After the removal of non-virion-associated biotinylation reagent by ultrafiltration, biotinylated viral vectors were conjugated to the surfaces of streptavidin-coated silica microbeads with a diameter of either 0.58 μm (5×10^{10} microbeads per ml) or 2.2 μm (9.8×10^9 microbeads per ml) (Bangs Labs). An equal amount of biotinylated viral vectors (6×10^8 viral particles in 50 μl) was combined with 0.5, 2, or 20 μl of a streptavidin-coated silica microbead suspension. This correlates to viral particle to microbead ratios of 24, 6, and 0.6, respectively, for the 0.58-μm-diameter microbeads, and of 120, 30, and 3, respectively, for the 2.2-μm-diameter microbeads. Analysis of the supernatants of these mixtures after centrifugation revealed no infectivity, indicating that the binding capacity of the streptavidin-coated microbeads for biotinylated adenoviral vectors was not exceeded under the viral particle to microbead ratios used. Adenovirus–microbead conjugates were washed with PBS (pH 7.6) supplemented with 0.5% Tween 20 (PBST) by three rounds of centrifugation at 5000 *g* for 3 min at 4°C, with each spin followed by resuspension of the conjugates in 1 ml of fresh PBST. Finally, adenovirus–microbead conjugates were resuspended in PBS without Tween 20 at final microbead concentrations of 1.25×10^6 microbeads/μl for the 0.58-μm-diameter microbeads and 2.4×10^6 microbeads/μl for the 2.2-μm-diameter microbeads. During these manipulations, no apparent loss of microbeads was seen by visual inspection.

The infectivity of these adenovirus–microbead conjugates was determined by using either D-17P4, C6, or COLO 205 cells as targets. Cells (5×10^4) were seeded in wells (15.5 mm in diameter) of a 24-well plate 24 h prior to infection. A suspension (1 or 5 μl after appropriate dilutions) containing adenovirus–microbead conjugates in PBS, which were fully dispersed by gentle pipetting, was placed over monolayers of cells (volume of culture medium, 1.0 ml per well) and incubated at 37°C. For control experiments, free adenoviral vectors in the same volume of PBS were added to cells and incubated

at 37°C. At 48 h postexposure, cells were fixed and stained for β-galactosidase (*LacZ*) activity by using X-gal as the substrate, as described above.

For the analysis of localized transduction of target cells by these conjugates, D-17P4 cells were plated in culture dishes (1×10^7 cells per 150-mm-diameter dish and 2×10^5 cells per 35-mm dish) 24 h prior to infection. A suspension (100 μl for the 150-mm-diameter dish and 24 μl for the 35-mm-diameter dish) containing adenovirus–microbead conjugates in PBS was placed over monolayers of cells (volume of culture medium, 12 ml for the 150-mm-diameter dish and 2 ml for the 35-mm-diameter dish) in the pattern of the letter "M" or "K." As a control, an equal number of free adenoviral vectors in 10 μl PBS was placed over cells. Cells were cultured at 37°C for 48 h and stained for the expression of the *lacZ* (β-galactosidase) gene using X-gal as the substrate, as described above.

Preparation and analysis of adenovirus–paramagnetic microparticle conjugates

Biotinylated adenoviral vectors (6×10^8 viral particles in 50 μl), prepared as above, were combined with a suspension of streptavidin-coated paramagnetic microparticles (2 μl containing approximately 1×10^6 paramagnetic microparticles with an average diameter of 1.0 μm; Promega). The mixtures were incubated at room temperature for 30 min with occasional mixing by gentle pipetting. Analysis of the supernatants (unbound fractions) of these mixtures after centrifugation showed no infectivity, indicating that all of the viral particles had been bound to streptavidin-coated paramagnetic microparticles (an average of approximately 600 viral particles per magnetic microparticle). Adenovirus–microparticle conjugates were washed by centrifugation of the mixtures and subsequent resuspension of the microparticle pellets in fresh PBST. Final adenovirus–microparticle pellets were resuspended in 100 μl PBST at a concentration of approximately 1×10^4 paramagnetic microparticles/μl. No apparent loss of adenovirus–microparticle conjugates was seen by visual inspection during these procedures. The infectivity of these adenovirus–microparticle conjugates was determined by titration on D-17P4 and C6 cells using the same method as for adenovirus–microbead conjugates as described above.

For analysis of the ability of these conjugates to be localized by magnetic force, D-17P4 cells (2×10^5) were placed in wells (35 mm in diameter) of a six-well plate 24 h prior to infection. A suspension (2 μl) containing adenovirus–microparticle conjugates in PBS was placed over monolayers of cells (volume of culture medium per well, 2 ml) and incubated at 37°C. For control experiments, the same number of free adenoviral vectors in 2 μl PBS was added to cells and incubated at 37°C. Small, rare-earth (neodymium-iron-boron) magnets (5 mm in diameter; 10,800 G; Tandy Corp., Fort Worth, TX) were taped to the bottom of the wells using standard masking tape, prior to the administration of adenovirus–microparticle conjugates. The plates were gently shaken using an orbital shaker at room temperature for 30 min, and then the magnets were removed from the plates. Cells were cultured at 37°C for 48 h and stained for the expression of the *lacZ* (β-galactosidase) gene using X-gal as the substrate.

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